Semiochemical mediated oviposition and mating in *Phlebotomus argentipes* (Diptera: Psychodidae) sand flies

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Submitted for the fulfilment of the requirements of the degree of Doctor of Philosophy.

July 2016

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Abstract

Phlebotomus argentipes (Diptera: Psychodidae) is an important vector responsible for the transmission of *Leishmania donovani* that causes visceral leishmaniasis (VL) or kala-azar, in the sub-continent of India. The aims of this study were to investigate the semiochemicals that mediate oviposition and mating behaviour and also the courtship behaviours in *P. argentipes*. The result of ovipositional behaviour bioassays shows gravid *P. argentipes* females preferred to oviposit their eggs in the present of conspecific eggs and also eggs extract. This suggests the presence of an oviposition pheromone on the surface of the eggs which can be removed by washing with an organic solvent and transferred to an alternative surface. A Y-tube olfactometer was used to test an upwind anemotactic response of virgin females to male headspace volatiles and male extract, in the presence or absence of host odour. The results strongly suggest that a volatile male-produced sex pheromone is present in *P. argentipes*. The results also suggest that under certain circumstances of the age of males and females and the presence of host odour, the females are attracted to live male and male extract of *P. argentipes*. Thus, presence of host odour might have a synergistic effect on the male-produced sex pheromone. Quantitative description and detailed of courtship behaviour(s) in both males and females of *P. argentipes* were observed. The results show that male behaviours during courtship are vital for the success of the mating. These predictor behaviours include approach wing-flapping, abdomen bending and copulation attempt by male *P. argentipes*. Understanding of the biology, ecology and chemical mediated behaviour in *P. argentipes* will enhance and widen the knowledge leading to the improved of the efficiency and efficacy of the current sand fly control programmes.

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Acknowledgements

I would like to thank and acknowledge:

- My supervisors; Prof. Gordon Hamilton and Prof. Richard Ward for their support, encouragement and supervision
- My advisor, Dr. Srabasti Chakravorty
- The Malaysian Ministry of Higher Education who funded my PhD scholarship
- The Universiti Malaysia Sarawak (UNIMAS) who is my employer that supported my PhD in Keele University
- The Postgraduate Administrator of Institute of Science and Technology in Medicine (ISTM), Ms Lisa Cartlidge who has been helpful and sweet during PhD
- Keele University School of Life Sciences staff, Ms Jayne Bromley, Ms Christine Bain, Ms Brenda Bould, Lisa Preston, Ian Wright, Alan Dudley, Nigel Bowers, Ms Debbie Adams and Mr. Dave Bosworth and others in assisting me in all sorts of things throughout my time in Keele
- All the Chemical Ecology Group members, Dr. Daniel Bray, Dr. Ifhem Chelbi, Dr. Pam Taylor, Ms Ann Underhill, Mr. Fraser Mitchell, for their continuous help and support with my work
- My wonderful colleagues, Dr. Pam Deavill, Dr. Tarek Mukassabi, Dr. Joseph Burhani, Dr. Abbey Olaniran, Clare and other postgraduate colleagues
- My family members, especially my mother, Hajah Dara Dobon and my late father, Allahyarham Haji Yaman Zaidil.

CHAPTER 1: GENERAL INTRODUCTION

1.1 INTRODUCTION

Sand flies are blood-sucking insects in the Order Diptera (Psychodidae: Phlebotominae). They are found throughout the tropical, subtropical and temperate regions of the world. They act as vectors of the etiologic agents of the medically important group of diseases known as Leishmaniasis which affects 12 million people in more than 88 countries (WHO, 2009). They also transmit other disease causing agents e.g. bacteria of the genus *Bartonella* (Birtles, 2001), phleboviruses (Tesh, 1988) and certain flaviviruses (Ashford, 2001). These cause significant problems in humans and domesticated animals, in some countries.

About 800 species of sand flies have been described and are divided into five widely accepted genera namely *Phlebotomus* and *Sergentomyia* in the Old World, and *Lutzomyia*, *Brumptomyia* and *Warileya* in the New World (Ward, 1985; Dedet and Pratlong, 2003). Approximately 70 species of sand flies are vectors of several species of *Leishmania* with about 40 species belong to the genus *Phlebotomus* and 30 species belong to the genus *Lutzomyia* (WHO, 1990).

Phlebotomine sand flies are distinct from other flies that are sometimes referred to as sand flies. Members of the families Simulidae or Ceratopogonidae can sometimes be referred to as sand flies because of their association with sandy habitats. The distinctive features that differentiate Phlebotomine sand flies from other subfamilies within the Psychodidae are the presence of biting mouthparts that are longer than the head, five-segmented palps, almost cylindrical antennae, a five-branched radial vein on the wing and the absence of an eye-bridge (Lane, 1993). Other general distinguishing features are their small body size (about 1.5-3 mm long), characteristic hopping flight and the 'V-shaped' position in which they hold their wings while resting (Killick-Kendrick, 1999).

Sand flies have a holometabolous life cycle, i.e. going through a developmental cycle in which the body form abruptly changes at the pupal moult. Female sand flies may lay up to a maximum 100 eggs in a single oviposition but normally it is about 40 eggs. The eggs are dark brown or black and elliptical in shape with an elaborate chorionic sculpturing. The variations in the patterns of these exochorionic sculptures on eggs could be used for species identification (Ward and Ready, 1977; Fausto *et al.*, 1991; Perez and Ogusuku, 1997). They are laid singly with the size of each egg being about 320 to 450 µm. The eggs are believed to be laid in microhabitats that are rich in organic nutrients but they are difficult to find in nature. The eggs of some species are able to undergo a diapause (delayed development) during long cold periods or during dry seasons (Johnson and Hertig, 1961; Ward and Killick-Kendrick, 1974). Eggs are typically laid in humid conditions and cannot survive prolonged desiccation. Eggs will hatch

into larvae within 4 to 20 days depending upon the temperature and the species of sand fly.

There are four larval stages, which feed on a variety of decaying organic materials thought to be present at oviposition sites. Sand fly larvae have long caudal setae, with one pair in L1 and two pairs in L2 to L4. Larval development takes approximately 20–30 days depending upon species and prevailing environmental conditions e.g. temperature and food supply. Diapause occurs during the fourth larval stage of several species inhabiting areas with cool winters. Induction of diapause in *Phlebotomus ariasi* is controlled by prevailing temperature while in *P. perniciosus* is controlled by temperature and photoperiod (shortening of day length) (Ready and Croset, 1980).

Before pupation, the fourth stage larvae empty their guts contents, and cease to feed and a visible thoracic swelling begins to appear. They attach themselves to the substrate using their terminal segment and moult into pupae, which at first are pale brown in colour and later become blackish. Pupae emerge into adult sand flies after 7 to 8 days.

The newly emerged adult sand flies first appear with crumpled wings, however within a few hours, they start holding their wings above their body at an angle of 45° relative to it. Both sexes of sand flies feed on sugar, which they obtain from plants or the honeydew of aphids (Young *et al*., 1980; Killick-Kendrick and

Killick-Kendrick, 1987; Schlein and Yuval, 1987; Mac Vicker *et al*., 1990; Schlein and Jacobson, 1999). Only adult females are haematophagous. In most of species of sand flies a blood-meal is essential to provide nutrition for the production of eggs, and sand flies are gonotrophically concordant (i.e. they take one blood meal per batch of eggs). Nevertheless some species undergo autogeny i.e. they have the ability to lay eggs without a blood meal (e.g. *P. papatasi*, (Schmidt, 1965; Benkova and Volf, 2007), *P. chinensis* (Guanghua *et al*., 1984, 1985), *P. gomezi, P. panamensis and P. sanguinarius* (Johnson, 1961)). Sand flies are pool feeders and feeding takes place on exposed parts of the hosts' body. The female sand fly thrusts her mouthparts into the skin and sucks the blood that accumulates. Males are ready to mate only after their genitalia have rotated through 180° and this normally takes place during the first 24h after emergence. Mating takes place before, during or after the females have taken their blood meals, whereas oviposition takes place 3 to 8 days after a blood meal.

Their short and hopping flight gives rise to the assumption that they do not disperse far from breeding sites. While this is true for some species e.g., *P. orientalis* was found between 45 - 730 m from the release point in a study done in Sudan (Quate, 1964), in Ethiopia, *P. longipes* flew as far as 240 m in one night (Foster, 1972). In another study in Panama, 20,000 flies were marked and released, the majority of the sand flies were recaptured within 50 m of the release point and only four were recaptured 200 m away (Chaniotis *et al.,* 1974). However, in southern France *P. ariasi* has been shown to fly as far as 2 km (Killick-Kendrick *et al*., 1984).

Sand flies are crepuscular or nocturnal insects. Their biting activities occur at different times of the night depending upon species, although a few species will bite during daylight when disturbed (Lane, 1993). Daytime resting places are comparatively cool and humid and include houses, latrines, cellars, stables, caves, fissures in walls, rocks or soil, dense vegetation, tree holes and buttresses, burrows of rodents and other mammals, bird's nests and termitaria (Killick-Kendrick, 1999).

Little is known of the natural breeding sites of sand flies but it is believed that the breeding sites can vary from domestic, peridomestic and sylvatic habitats (El Naiem and Ward, 1991; Killick-Kendrick, 1999; Alexander and Maroli, 2003; Feliciangeli, 2004). Feliciangeli (2004) described finding *Phlebotomus mascittii* (Grassi 1908) larvae in a cellar in Rome in 1907. This was the first report of an immature stage of a Phlebotomine sand fly in nature. In the New World, the first finding of Phlebotomine breeding sites was by Ferreira *et al*. (1938), where four larvae were found at the base of a tree in Brazil and a dozen larvae in the wall of a house in Venezuela (Pifano, 1941). Feliciangeli (2004) suggests that, based on the frequency of the collections and the abundance of specimens caught, few sand flies have consistent breeding sites. For instance *P. papatasi* breeds in human dwellings and cattle sheds in India and gerbil burrows in Central Asia (Perfil'ev, 1968); animal burrows are used by *P. duboscqi* (Mutinga *et al*., 1986); and for *P. martini*, animal burrows and termite hills are the breeding sites in Kenya (Mutinga *et al.,* 1989).

1.2 LEISHMANIASIS

Leishmaniasis is caused by parasitic Protist of the genus *Leishmania*, order Kinetoplastida and family Trypanosomatidae. There are two principal forms of the Leishmania Protist that can be identified as distinct morphological stages; a rounded amastigote living and dividing in the macrophages of the vertebrate host, and a flagellated promastigote in the intestinal tract of the insect vector (Lainson and Shaw, 1987; Lainson *et. al*., 1987).

The genus *Leishmania* consists of 30 species, of which about 20 cause disease in human all are either zoonotic or have recent zoonotic origins (Ashford, 2000). All of the human diseases are known as leishmaniasis. Different species of *Leishmania* cause different diseases and different clinical manifestations of the disease in infected humans in various parts of world (Ashford, 1996).

Leishmaniasis occurs in 88 countries in four continents with 350 million people at risk and there is evidence that it is spreading. The World Health Organisation (WHO) has reported that worldwide, approximately 12 million people are presently infected, with 2 million new cases (1.5 million cutaneous leishmaniasis (CL) and 500,000 visceral leishmaniasis (VL)), occurring annually (Desjeux, 1996; WHO, 2009). The incidence of leishmaniasis is not evenly distributed in the endemic areas: approximately 90% of cutaneous leishmaniasis (CL) cases occur in seven countries i.e. Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria; whilst 90% of visceral leishmaniasis (VL) cases occur in

rural and suburban areas of five countries i.e. Bangladesh, Brazil, India, Nepal and Sudan (Gramiccia and Gradoni, 2005; WHO, 2009).

Leishmaniasis is endemic in areas of the tropics, subtropics, and southern Europe, in different locations varying from rain forests in the Americas to deserts in western Asia, and from rural to periurban areas (Ward, 1985; Herwaldt, 1999). Indeed, both human and animal leishmaniasis show a wider geographic distribution than before. Several cases of autochthonous *Leishmania* transmission have recently been reported from areas previously considered non-endemic, for instance in Nan Province and Bangkok in Thailand (Kongkaew *et al*., 2007; Maharom *et al*., 2008), western Upper Nile in Sudan (Desjeux, 2001) and the Northern Territory of Australia (Rose *et al*., 2004).

The forms of human leishmaniases are primarily based on the clinical presentation in infected persons; most notable are visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and muco-cutaneous leishmaniasis (MCL) (Desjeux, 1996; Dedet and Pratlong, 2003; WHO, 2009). As the disease progressively develops over time or is interrupted by treatment, this may lead to different clinical manifestations for example, post-kala-azar dermal leishmaniasis is manifested by various types of skin lesions and most prominently on the face, observed in patients recovering from VL, in East Africa and India (Rashid *et al*., 1986; Rees and Kager, 1987; Grevelink and Lerner, 1996; Herwaldt, 1999). Another type of VL is viscerotropic leishmaniasis, which is more typically

dermotropic (Magill *et al*., 1993). Leishmaniasis recidivans or recurrent leishmaniasis refers to the development of new lesions in the centre or periphery of a scar healed acute lesion of CL (Grevelink and Lerner, 1996; Herwaldt, 1999).

In cutaneous leishmaniasis (CL), skin ulcers formed at the site of sand fly bites on exposed areas such as the face, arms and legs and this may lead to disfiguring scars on the patients. In muco-cutaneous leishmaniasis (MCL), there are progressively destructive ulcerations of the mucosa, extending from the nose and mouth to throat cavities and surrounding tissue. Diffuse cutaneous leishmaniasis (DCL) is an anergic variant of CL in which lesions are disseminated, resembling lepromatous leprosy, in other areas of the skin such as limbs, buttocks and face. Visceral leishmaniasis (VL), is characterised by high fever, substantial weight loss, anaemia and swelling of the spleen and liver and is fatal if left untreated. There are two types of VL; zoonotic VL, transmitted by a vector from animal to human; and anthroponotic VL transmitted by a vector from human to human.

The diversity in the clinical syndromes of leishmaniasis is observed partially because there is a difference in the infecting species of *Leishmania* involved in each type. In CL, *Leishmania major*, *L. tropica, L. infantum, L. brazieliensis*, and *L. mexicana* are proven to be involved, while in mucocutaneous, *L. brazieliensis* and *L. infantum* are involved. In VL, *L. infantum* is involved in zoonotic VL in Europe and South America, whereas *L. donovani* is involved in anthroponotic VL in Asia

(Grevelink and Lerner, 1996; Killick-Kendrick, 1999; Ashford, 2000; Gramiccia and Gradoni, 2005).

1.3 *PHLEBOTOMUS ARGENTIPES* (DIPTERA: PSYCHODIDAE)

Phlebotomus argentipes Annandale and Brunetti (Diptera: Psychodidae) a sand fly of the Old World has a wide distribution in most Asian countries namely: India, Bangladesh, Borneo, Burma (Myanmar), Indonesia, Laos, Nepal, Pakistan, Sri Lanka, Thailand, Vietnam, West Malaysia and Iran (Lewis, 1978, 1982). Although it has been found over a wide geographical range in Asia, visceral leishmaniasis (VL) is confined to North Eastern and Southern India and neighbouring Nepal and Bangladesh (Ilango *et al*., 1994; Ilango, 2000).

P. argentipes is the only proven vector for VL in India and *Leishmania donovani* has successfully been transmitted by the bite of *P. argentipes* to human volunteers (Swaminath *et al.*, 1942). The first natural infection was reported in a single female of this species caught in an endemic area in North Bihar, India (Shortt *et al*., 1926, 1929). In Bangladesh, India and Nepal, it is estimated that 200 million people are at risk of human VL. It has been reported that the disease occurs in more than 109 districts on the Indian sub-continent, where it affects mostly the poorest people (Surendran *et al.,* 2005; WHO, 2006; Das *et al*., 2008). In India, it is estimated that 100,000 VL cases occur annually. More than 90% of the cases are reported in Bihar State (Singh *et al.,* 2006). Whilst in Bangladesh, it is estimated that approximately 40,000 to 45,000 cases occur annually, with a

population of 20 million people at risk (Bern and Chowdhury, 2006). In Nepal, VL cases are on the rise, with a sharp increase in the population at risk of 5.6 million people in 2006 to eight million in 2007. It was estimated that by 2008, one third of the Nepalese population (total of 29 million) would be at risk of contracting the disease (Anon, 2008). There have also been confirmed cases of Kala-azar reported in Afghanistan.

P. argentipes occurs in domestic and peridomestic environments, and is almost endophagic and endophilic in cattle sheds and houses (Hati *et al*., 1980; Hati, 1983, 1991). Adults were also reported to have been caught in; tree holes, wells, culverts, under scrubby vegetation and in caves (Kaul *et al*., 1979; Apiwathnasorn *et al*., 1993). Recently, investigations of the breeding ecology of immature stages of this species have been carried out using the soil incubation method (a collecting method for immature stages) in Bihar State, India. The findings showed that *P. argentipes* breed more in the cattle sheds than in human houses, which appears to be associated with the pH of the soil i.e. alkaline soil (Singh *et al*., 2006).

Blood meal analyses of *P. argentipes* indicated that this species feeds on cattle when they are available and that humans are a secondary choice for feeding and it has been suggested that the presence of animals reduce the man-vector contact (Dhanda and Gill, 1982; Dhiman *et al*., 1984; Pandya, 1985; Mukhopadhyay and Chakravarty, 1987; Palit *et al*, 1988). Dinesh *et al.* (2001)

reported that the landing/biting on man varied in different seasons. It was found that the number of *P. argentipes* caught was highest in summer, followed by the rainy season and then lowest during winter and abundance seems to be inversely correlated with rainfall.

P. argentipes has shown geographical variation (Lewis, 1957). It has been observed that in India, it is anthropophilic and a vector for VL whereas in Sri Lanka, it is zoophilic (Lewis and Killick-Kendrick, 1973). It has been suggested that it may be a species complex where a zoophilic and anthropophilic species can occur in the same area (Bray, 1974). Lewis (1978) however rejected the proposal that two species of *P. argentipes* occur in India and that *P. argentipes* is a species complex. The only distinctive feature between the 2 proposed members of the species complex is the difference in the length of ascoids on the antennae; the sand fly with short ascoid (anthropophilic) can be found in eastern India and the long ascoids form (zoophilic) in South-east Asia (Lewis, 1978).

In recent studies, it was found that VL distribution is correlated with the length of the sensilla chaetica. Additionally, *P. argentipes* sand fly populations in India are reported to be sympatric (Lane and Rahman, 1980; Ilango *et al*., 1994) and variation in cuticular hydrocarbons of the species has been reported (Kamhawi *et al.*, 1992). These studies suggest that *P. argentipes* is a species complex, with two morphologically distinct species with differing vectorial capacity (Ilango, 2000; Surendran *et al.,* 2005). It is proposed that variation in feeding

preferences and morphology of the vector is the cause for the limited range of VL transmission relative to the vector distribution. As VL is believed to be an anthroponosis and humans are the reservoir, hence only anthropophagus *P. argentipes* are susceptible to *L. donovani*. This is consistent with the work of Lewis and Killick-Kendrick (1973) which reported that *P. argentipes* was almost entirely zoophilic in its feeding behaviour in the southern part of its range (Lane and Rahman, 1980; Kamhawi *et al*., 1992; Ilango, 2000; Surendran *et al.,* 2005).

1.4 SEMIOCHEMICALS

Semiochemicals are the chemical signals used for communication between two organisms. They are emitted by one individual and cause a behavioural response in another (Law and Reigner, 1971). Volatile semiochemicals are perceived by olfaction and involatile semiochemicals are perceived by contact chemoreception.

Two categories of semiochemical, attractant and repellent, refer to behaviour modifying olfactory compounds that do not require contact with the source. Behaviour modifying chemicals that are active on contact or at close range are stimulants and deterrents (Dethier *et al*., 1960; Foster and Harris, 1997).

Semiochemicals are described as information conveying chemicals and toxins that have been used as chemical communication between interspecific and intraspecific organisms (Norlund and Lewis, 1976; Norlund, 1981). They convey

information, that have been grouped into three classes i.e. 1) depending on the interaction, whether it is an intraspecific or interspecific; 2) the cost and benefit that may fall to each organism; and 3) the identity of the producer and receiver (Dicke and Sabelis, 1988). Thus, an info-chemical is a substance that conveys information between two organisms in which the sender releases a chemical substance that evokes a behavioural or physiological response from the receiver.

An allelochemical is an info-chemical that mediates an interaction between two organisms of different species. It was categorised by the cost and benefits of the organism that has sent the signal (emitter), and the organism that has received the signal (receiver). The four categories are, 1) allomones (which favour the emitters and not the receivers); 2) kairomones (which favour the receivers and not the emitters); 3) synomones (which benefit both emitters and receivers); and 4) apneumones (which are derived from non-living sources) (Hick *et al*., 1999). An allomone is defined as a chemical substance, produced or acquired by an individual which when received by another individual from different species in a natural context, evokes a behavioural or physiological reaction from the receiver that is adaptively favourable to the emitter. In contrast, a kairomone is a trans-specific chemical messenger, which benefits the recipient rather than on the emitter (Brown, 1968).

Pheromones are a subclass of semiochemicals, pheromones mediate an interaction between two organisms of the same species (intraspecific chemical

signals). The word pheromone is derived from the Greek *pherein* = to transfer or carry and *hormone* = to excite or stimulate. It has been defined as ‘a substrate secreted to the outside by an individual and received by a second individual of the same species in which it releases a specific reaction i.e. a definite behaviour (releaser pheromone) or developmental process (primer pheromone)’ (Karlson and Lüscher, 1959). Pheromones are usually divided by function for examples oviposition pheromone and sex pheromones. In Diptera, oviposition pheromones are used by gravid (blood-fed) females to find a suitable location to oviposit and sex pheromones are used by females to find and choose a mating partner of the right species, sex and reproductive stage.

The importance of pheromones in nature has been recognised and they have been used to manipulate the behaviour of animals. Now, pheromones are extensively used for insect pest management, with significant cost and environmental benefits i.e. they are safer compared to insecticides and specific to the target species (Minks and Kirsh, 1998). The main ways of exploiting pheromones to control insects are monitoring, mating disruption, mass trapping (lure-and-kill) and other manipulation methods. In monitoring, pheromone-based baited traps provide one of the most effective methods for the surveillance of a target insect, even when the population levels are very low and provide an early warning allowing timely interventions for insect control (Wall, 1989). Mating disruption is use to control insects by causing communication disruption between adult males and females to find each other, thus reduce mating and indirectly stopping the reproduction of the next generation (Campion, 1983; Carde and

Minks, 1995). Mass trapping or ‘lure-and-kill’ (also known as attracticide) control is use to reduce an insect population by attracting insects with pheromones and then either trap or kill the insects (McCall and Cameron, 1995).

1.5 SEMIOCHEMICALS IN SAND FLIES

Studies on the chemical ecology of phlebotomine sand flies have been pursued because of their importance in the transmission of Leishmaniasis. Most of the studies are related to the use of chemical cues by sand flies to survive in the environment. These include 1) sex pheromones which are used by adult sand flies to find mating partners; 2) oviposition pheromones which are used by gravid females to locate suitable (available food for the immature stages) places to oviposit and also 3) kairomones such as host volatile chemicals to attract sand flies to target locations.

The initial evidence of the existence of an oviposition pheromone was from the eggs of *Lutzomyia longipalpis*. This was demonstrated when gravid females were shown to prefer to oviposit at sites where conspecific eggs were already present (El-Naiem and Ward, 1990). They later showed that the age of the eggs was unimportant in eliciting a response but the number of eggs present at the site was crucial. They showed that although 40 eggs were not sufficient to induce a response from gravid females, 80 - 320 eggs elicited a strong response. They also demonstrated that the attractant effect of the eggs could be removed by washing them in organic solvents and water (El Naiem and Ward, 1991). Dougherty *et al.*

(1992) demonstrated that ovipositing females were attracted to a hexane extract of conspecific eggs and that the volatile compounds identified on the external surface of the eggs were identical to those found in the accessory glands of the females. A hexane extract of eggs elicited a positive oviposition response from the gravid females.

Identification of the active component of the extract was achieved by fractionating the whole egg extract by high performance liquid chromatography (HPLC) and then testing the fractions to see which one was responsible for attracting gravid females to oviposit (Dougherty *et al*., 1994). The active semiochemical fraction was found to attract gravid females thereby increasing the number of eggs laid in the vicinity of the eggs that were already present. Dougherty *et al.* (1994) also demonstrated that ovipositing *Lu. longipalpis* females produce the oviposition pheromone in the accessory glands. The pheromone coated the surface of the eggs as they passed through the oviducts during oviposition. The oviposition pheromone was identified by gas chromatography-mass spectrometry (GC-MS), gas chromatography (GC) and chemical derivatisations as the C12 fatty acid, dodecanoic acid (Dougherty and Hamilton, 1997).

In nature, environmental oviposition cues combined with the oviposition pheromone may assist female sand flies to locate a suitable oviposition site with sufficient food for their immature stages to develop (McCall and Cameron, 1995).

Experiments that have been carried out in the laboratory demonstrated the presence of environmental cues such as organic materials which influence gravid females in choosing suitable oviposition sites.

El Naiem and Ward (1992a) carried out experiments using frass (faecal remains), larval rearing medium and rabbit faeces to determine the attractiveness of organic materials to gravid females. They found that these organic materials attracted ovipositing sand flies. They also showed that these organic materials stimulated females to oviposit earlier and increased post-oviposition survival. In addition, they showed that extracts of organic materials, made in polar and non-polar solvents, for example rabbit food and oviposition pheromone produced a synergistic effect that resulted in greatly increasing the numbers of eggs laid and a highly focused response. Oviposition survival was increased X3.5 and the numbers of eggs laid increased by X2.5 (Dougherty *et al*., 1993). Dougherty *et al.* (1993) showed that combination of dodecanoic acid (oviposition pheromone) and also hexanal and 2-methyl-2-butanol (oviposition attractant apneumones from rabbit faeces) in a bioassay, enhanced oviposition by the ovipositing sand flies. It was suggested that sand flies acquired hexadecanoic acid (palmitic acid) from the blood meal and then converted it to dodecanoic acid.

The situation regarding the presence of oviposition pheromones in other species of sand flies e.g. *Phlebotomus papatasi* (Scopoli, 1786) is less clear. Srinivasan *et al*. (1995) showed that gravid females were attracted to conspecific

eggs. They also found that the presence of 100–200 eggs was highly attractive to gravid females while the presence of 10-40 eggs in the vicinity elicited no oviposition response. The extract of conspecific eggs using diethyl ether showed a positive oviposition response from gravid females of *P. papatasi.* These findings are similar to those of *Lu. longipalpis.*

It has been demonstrated that male *Lutzomyia longipalpis* sand flies produce sex pheromone to attract female sand flies to a mating site. There is also evidence that the sex pheromones are attractive to their males. Male sex pheromones are produced in the papule-like structures on pale cuticular patches of the tergites of males of that species (Phillips *et al*., 1986; Ward *et al*., 1989, 1991; Lane and Bernades, 1990; Hamilton *et al*., 1994). At first, male spot patterns were thought to be the markers associated with two possible sibling species. From cross-mating experiments, the difference in the male spot pattern was found to be associated with pre-zygotic mating barriers and it was suggested that *Lu. longipalpis* is a sibling complex. However cross-mating studies carried out by Ward *et al*. (1991) showed that the cross-mating barriers were not related to the number of spots directly. The two male spot patterns are a single pair of tergal spots on abdominal segment IV (1S males) and additional pair of tergal spots on segment III (2S males) (Ward *et al*., 1991). Nonetheless, the male tergal spot is useful as a morphological indicator of different pheromone producing types in some parts of Brazil (Ward *et al*., 1983, 1988; Lanzaro *et al*., 1993, Arrivilaga and Feliciangeli, 2001; Hamilton *et al*., 2005; Souza *et al*. 2008).

The structure of the pale spot patches is characterised by numerous small cuticular papules. Underlying these are large vacuolated cells with a complicated end apparatus, surrounded by an area of highly convoluted cell membrane which forms microvilli, opening into a central reservoir. The reservoir is connected to the surface of the insect by a small cuticular duct. The physical appearance of the cells, ducts and papules led workers to believe that it was a secretory cell that secretes sex pheromone. Analysis of compounds extracted from the tergal glands showed that there were “pheromone-like” substances present in the cells with molecular weight of either 218 ($C_{16}H_{26}$) or 272 ($C_{20}H_{32}$). These were identified as farnasene-like and diterpenoid-like structures respectively (Lane *et al.*, 1985; Phillips *et al*., 1986; Lane and Bernades, 1990).

Later, Hamilton *et al.* (2002) showed that terpene sex pheromones are not widely distributed amongst male *Lutzomyia* sand flies and the abdominal papules seen on males of some *Lutzomyia* species maybe non-functional. Furthermore, they have confirmed that males of the New World sand flies that do not possess papules will not secrete terpene sex pheromones. It has been suggested that the differences in quantities and qualities of terpene extract in male sex pheromones of members of *Lu. longipalpis* from different chemotypes represent significant taxonomic differences (Hamilton *et al.*, 1994; Arrivillaga and Feliciangeli, 2001).

At present, there are five different sex pheromone-producing populations (chemotypes) of members of the *Lu. longipalpis* species complex that have been

established from six regions in Brazil based on terpene component of their sex pheromone (Hamilton *et al*., 2005). They have been shown to produce three distinct pheromones, which can be distinguished by qualitative differences in the major terpene component: 1) a C16 (m.w. 218) bicyclic homosesquiterpene, 3-methyl-α-himachalene [3MαH]; 2) a C16 (m.w. 218) monocyclic homo-sesquiterpene, (*S*)-9-methyl-germacrene-B [9MGB] (Hamilton *et al*., 1996a,b), and 3) a C20 monocyclic diterpene, partially characterised as a cembrene yet to be structurally fully elucidated (Hamilton *et al*., 2004, 2005; Hamilton, 2008). Other species in the subfamily that have been examined are *Lu. lenti* and *Lu. pessoai* which were found to use diterpenes as sex pheromones. In addition, *Lu. cruzi* has also been shown to secrete a homosesquiterpene whilst in *Lu. lichyi*, is believed to produce an oxygenated form of homosesquiterpene. Finally, *Lu. carmelenoi* was found to secrete sesquiterpenes as sex pheromones (Hamilton and Ward, 1994; Hamilton *et al.,* 1999, 2002).

The dynamics of sand fly aggregation and orientation to host animals are not completely understood. Jarvis and Rutledge (1992) described the “lek-like” behaviour of adult male *Lu. longipalpis* sand flies which has been observed in the laboratory and field. These authors considered that male age as well as the aggressive jostling between them are important factors in successful mating. A lek is defined as a male mating aggregation associated with a defended territory that contains no resources other than available courting males, which females visit for the purpose of mating (Bradbury, 1981; Jones and Quinnell, 2002). The aggregation behaviour of *Lu. longipalpis* starts when males are attracted by the

odour of host animals. Then the pheromone produced by the first male to arrive may act as an additional attractant for male and female sand flies (Quinnell and Dye, 1994). As a consequence females are attracted and other males are recruited to these feeding and mating sites (Dye *et al*., 1991; Quinnell and Dye, 1994; de Melo Ximenes *et al*., 1999).

In the Old World sand fly species, *Phlebotomus argentipes*, similar lek-like male aggregation behaviour has been observed in the field. Males arrive first, early in the evening on a selected host cow and beat their wings vigorously and jostle with each other, a large aggregation of evenly spaced males is assembled and then smaller numbers of females arrive later in the evening for reproductive and feeding purposes (Lane *et. al*., 1990). In addition, Lane *et al.* (1990) noted that swarms were observed clearly on cattle (where hundreds of flies may be involved) and smaller aggregations were seen on humans, walls or other vertical surfaces adjacent to cattle in a manner similar to other aggregating and sex pheromone producing species e.g. *Lu. longipalpis*. To date, no chemical or biological evidence for sex or other pheromones have been found in this species. In other species e.g. *P. duboscqi* a distinctive behaviour has been observed in which an adult male was reported to ‘piggy back’ their female mate by clasping her abdomen with his coxites. This behaviour was believed to be a form of courtship rather than mate guarding, and enables the female to recognise a potential mate (Valenta *et al*., 2000). These observations suggest that perhaps different mating strategies are employed in different species of sand flies. Thus it would be interesting to carry out a similar study in different species of sand flies.

Phlebotomine sand flies respond to host odour kairomones in laboratory and field studies e.g. female sand flies use host odour kairomones to locate a suitable host (Nigam and Ward, 1991; Oshaghi *et al*., 1994; Dougherty *et al*., 1999). Laboratory and field work proved that female sand flies have the ability to discriminate between the different types of hosts that may be available and have a tendency to feed on one selected host from many (Morrison *et al*., 1995). Hence, it has been suggested that the initial male sand fly host choice is based on the host odour components present and subsequent female and male choice is based on the interaction of host odour components and male sex pheromone. A host odour contains a mixture of attractive and repellent elements and attraction to a host animal is likely to be based on the balance between these different categories of kairomones in the animal's odour profile (Hamilton, 2008). Hamilton and Ramsoondar (1994) demonstrated that female *Lu. longipalpis* were differentially attracted to human hosts based on the odours of the hosts, a phenomenon that has been well documented in mosquitoes (Takken, 1991; Qiu *et al*., 2006).

Carbon dioxide is believed to play an important role in mosquito host-seeking behaviour. It has been shown to activate and attract several mosquito species (Takken, 1991) and is also to synergise the attractiveness of other host odour components e.g. *Aedes aegypti* (Dekker *et al*., 2005).

In the field, Pinto *et al.* (2001) showed that a combination of carbon dioxide and human kairomone is important for the attraction of *Lu. intermedia* and *Lu.*

whitmani. However in laboratory experiments, removing carbon dioxide from preparations of host compounds and sex pheromone did not reduce their attractiveness (Nigam and Ward, 1991). Recently, Bray and Hamilton (2007) demonstrated that 1% of carbon dioxide in air was attractive to female *Lu. longipalpis* but its presence did not synergise the attractiveness of sex pheromone. Moreover the study showed that males were only attracted to the combination of carbon dioxide and sex pheromone but not to each compound (Hamilton, 2008). Hamilton (2008) suggested that the interaction between host odour and sex pheromone is complex and possibly may depend on concentrations of the components of the odour.

At present, most of the work on semiochemical mediated oviposition and mating in Phlebotomine sand flies (Diptera: Psychodidae) has been done on *Lu. longipalpis*. By comparison *Phlebotomus argentipes* Annandale and Brunetti, arguably the most important vector of Leishmaniasis has received very little attention and no chemical or biological evidence for pheromones has been presented apart from the reports of aggregations on host animals. A comprehensive understanding of the chemical ecology of this vector will enable us use this information to develop new tools for monitoring and control of the insect in the future that may lead to new disease control options.

The aim of this study was not only to gather information on semiochemical-mediated oviposition and sex pheromone production in *P. argentipes*, but also to

provide baseline data to inform the potential use of sand fly pheromones in the development of novel traps and control strategies in the vector control programme of visceral leishmanisis.

1.6 AIMS

The overall aim of this study was to investigate the semiochemical mediated oviposition and mating behaviour of *Phlebotomus argentipes*.

The objectives of the work:

1. To determine if oviposition pheromone is present on eggs.
2. To determine if the oviposition pheromone is coated on the surface of eggs.
3. To determine if sex pheromone is present in *P. argentipes* virgin males using a Y-tube olfactometer.
4. To establish the age when sex pheromone is produced in males and the age of females that are most responsive to sex pheromone in *P. argentipes.*
5. To determine the effect of sex pheromone of *P. argentipes* virgin males in the presence of host odour in a Y-tube olfactometer.
6. To determine if sex pheromone can be extracted from *P. argentipes* males in hexane solvent.
7. To describe in detail the courtship behaviour of *P. argentipes* males and females during mating.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 INSECT MAINTENANCE

The Keele University *Phlebotomus argentipes* colony was established from pupae supplied in September 2010 by the Walter Reed Army Institute of Research (WRAIR), USA and has been maintained at Keele University since then. It was maintained using a combination of methods described by Modi and Tesh (Modi and Tesh, 1983) and Ghosh and Bhattacharya (Ghosh and Bhattacharya, 1989). The immature stages (eggs, larvae and pupae) were maintained in an environmental chamber (Versatile Environmental Test Chamber; Sanyo, MLR-351/MLR-351H; Figure 2.1) at 28°C±2°C and 12:12 (light:dark) photoperiod. Adults were maintained in an insectary at 27°C±1°C and a 12:12 (light:dark) photoperiod. A relatively high humidity of approximately 50-80% (r.h.), was consistently maintained for both parts of the colony by placing paper towels dampened with distilled water in both the adult holding cages and also the boxes containing the oviposition and larval pots. For sand fly rearing, careful control of both temperature and relative humidity play an important role in the success of the colony as well as the speed with which the sand flies progress through their developmental stages. Furthermore, to avoid fungal and mite contamination the larval pots were checked regularly and food and sand were added as required.

Left Right

Figure 2.1: The *Phlebotomus argentipes* immature stages were kept inside a Sanyo MLR-351/MLR-351H environmental chamber set at a temperature of 28°C±2°C and 12:12 (light:dark) photoperiod (left); the larval and oviposition pots were kept in plastic boxes which were in turn kept inside black plastic bags (right).

2.1.1 Adults

Adult flies were kept in (20 x 20 x 20 cm) nylon Barraud cages (Figure 2.2). They were allowed to feed on a 60% sugar (fructose) solution soaked onto a piece of cotton wool placed on a plastic vial cap at the bottom of the cage. The paper towels used to maintain humidity were hung over the metal frame used to support the Barraud cage on two sides of the cage. The cage was kept in a plastic bag to maintain a relatively high level of humidity at 60-80% r.h.

Emerging adult flies were transferred from the rearing pots to the nylon Barraud cage using a mechanical aspirator. Approximately 300-500 sand flies, including males and females, were kept in the cage to facilitate mating in preparation for blood feeding. Female flies aged 2-3 days old (d) were blood fed on anaesthetised mice twice a week. The mice used were about 6-8 weeks old and were anaesthetised by injecting a solution of Midazalom and Hypnorm (ratio=0.23:0.05 ml). Anaesthesia was administered by a UK Home Office registered personal licence holder. The anaesthetised mouse was left for about 3 minutes post injection to allow the drugs to take effect. To facilitate blood feeding, an area of the mouse's back was shaved, before putting it into a cage for blood feeding. The sugar solution was removed and the mouse was placed inside the cage on its abdomen. Blood feeding was allowed to take place over a period of approximately 1 hour, after this time, before the mouse recovered from the anaesthesia, it was subject to a schedule 1 euthanasia which again was carried out by a personal licence holder (UK Home Office Protocol, Section 19) under Project License 40/3279. The fully engorged blood-fed females, with a relatively

lower number of males to ensure that all females copulated, were allowed to remain in the feeding cage to complete defecation and oogenesis before they were transferred to oviposition pots after 3 days when gravid. Unfed females and excess males were transferred into another feeding cage.

Figure 2.2: Adult *P. argentipes* were kept inside Nylon Barraud cages (20 x 20 x 20 cm) which were supported on wire frames. Sections of blue paper roll were dampened with distilled water and hung on the wire frame on either side of the Barraud cage to maintain humidity. The cages were then placed inside plastic bags and kept in the insectary at 27±1°C and a 12:12 (light:dark) photoperiod.

2.1.2 Immature Stages

Oviposition and rearing pots were made of polymethylpentane (PMP) (Nalgene, BDH Chemicals) and were 11 cm in diameter and 7 cm in height with a base and screw cap lid. A hole, 8 cm in diameter (Figure 2.3), was cut out of both the base and the lid. The base was placed on a paper towel and a layer of Plaster of Paris was poured into the pot to a depth of 1-2 cm, to produce a solid base. Drawing a spatula gently through it before it had completely hardened created an uneven top surface in this base layer. This process created a series of grooves and indentations which facilitate better oviposition. A thin layer of plaster of Paris was also used to coat the wall of the rearing pots (Chelbi and Zhioua, 2007; Chelbi *et. al*., 2011). The top of the pot was covered with nylon netting held in place by the screw cap lid to prevent adult flies from escaping. A small incision (1-1.5 cm in length) was made in the Nylon netting and a piece of cotton wool was placed into the incision to plug the resultant hole (Figure 2.4). The hole was used as an access point for the aspirator to transfer adult flies into the pots. Only gravid females were transferred as the males may disturb the resting and ovipositing females.

The gravid females were kept in the oviposition pots for 5-6 days post blood meal until most of them had oviposited and died. After that their dead bodies were removed from the pots to avoid fungal contamination that would increase mortality of the eggs with fine forceps. Most of the eggs were deposited in the crevices on the damp plaster. A small quantity of larval food was sprinkled over the eggs in the pots as soon as the first sign of hatching was observed. The developmental stages

(eggs, larvae and pupae) took place within the same rearing pot and they were checked every day to for signs of contamination e.g. fungus that could increase the fatality of the immature stages in the pot. Larval food was added every other day or as appropriate. The pots were placed in a large plastic box (13 x 13 x 14 cm) (Figure 2.5) on paper towels that had been dampened with distilled water. These dampened paper towels and Plaster of Paris maintained high humidity in the boxes the humidity was maintained by adding water to the towels when they dried out.

The pots were labelled with the date on which the females had been blood-fed. Adult flies usually started to emerge 4-6 weeks after eggs had been deposited in the pots. When the larvae pupated a small piece of cotton wool soaked with 60% fructose solution was put under the rim of the screw cap lid between the nylon netting and the plastic of the lid ready for the new emerged adult flies to feed on. The pots were maintained in strict date order and as the adults emerged they were transferred from the rearing pots into the adult holding cage. Pots were checked on a daily basis. The pots were disposed of typically 8 weeks after the gravid females had been added by which time most of the pupae had become adults. Expired pots were carefully disposed of, Plaster of Paris was removed from the used pots, which were washed, dried and autoclaved prior to reuse. Care was taken to ensure correct disposal of used materials to avoid contamination.

All nylon netting materials, used for adult and immature rearing, were soaked in water with one or two tablets of Presept™ (Advanced Sterilization

Products; Johnson & Johnson, CA, USA), disinfectant solution for sterilising overnight. Then they were washed a few times with water and dried ready for their next use.

Figure 2.3: Shows a PMP rearing pot with a base layer of plaster of Paris which has been slightly roughened. The screw-cap lid is shown without the netting.

Figure 2.4: Shows a rearing pot containing recently emerged adult *P. argentipes*. The incision made in the netting covering the top of the pot to allow removal of adult sand flies can be seen plugged with a small ball of cotton wool.

Figure 2.5: Shows part of a plastic box (13 x 13 x 14 cm) used to keep oviposition and larval rearing pots in the Sanyo environmental chamber.

2.1.3 Larval Food

The larval food consisted of a blended mix of 200 g of fine silver sand (Bradstone, Derbyshire, UK), 200 g of John Innes potting compost No.2 (Westland Horticulture Ltd., UK), 210 g of *Daphnia* (Supa Aquatic Supplies Ltd, Sheffield, UK) and 200 g of guinea pig food. The potting compost and the fine silver sand were dried until they became free flowing, in order to avoid contamination later. When properly dried, both sand and potting compost were sieved before adding to the mixture of blended *Daphnia* and guinea pig food. The mixture was stirred well and ground in a coffee grinder to make up a fine larval food. Blended food was then put into bottles and autoclaved before use.

2.2 CLEANING OF BIOASSAY APPARATUS

All apparatus used in the bioassays were thoroughly washed to ensure they were clean and free from any contaminants. The presence of any contaminants, even in a small amount could potentially bias the response shown by the respondents in the bioassays.

2.2.1. Treatment of Glassware

All the glassware was washed with water before being immersed in 10% Teepol L detergent (BDH, Poole, UK) for at least an hour. It was then rinsed thoroughly with large amounts of water, followed by distilled water. It was then rinsed with acetone (Laboratory Reagent Grade; Fisher Scientific, Loughborough,

UK) was used to remove any water left on the glassware. Finally, all the glassware was baked in an oven overnight at 180°C.

2.2.2 Treatment of Teflon Items and Nylon Netting

All the Teflon items and nylon netting were also first washed with water and then immersed in 10% Teepol L detergent for at least an hour. The items were then washed thoroughly with water, followed by distilled water and lastly with acetone. Then, they were left to dry in a fume hood before they were used.

2.3 PREPARATION AND STORAGE OF CHEMICALS

Extracts were stored in a vials prepared from Pasteur pipettes (Scientific Laboratory Supplies). Vials were prepared by flaming a pipette over a Bunsen burner to drive off any contaminants. It was then sealed at the narrow end and left to cool down before use. Extracts were transferred into the vials, which were then sealed at the open end by flaming over a hot Bunsen burner. Vials were stored at approximately -20°C in freezer until use.

2.4 STATISTICAL ANALYSIS

The statistical Package for Social Science (SPSS) for Windows version 21 and Minitab 16 English version were used to analyse the data according to suitability of the statistical test.

For the analysis of the oviposition results, Minitab 16 (English version) was used. The data were entered and the variables were tested for their distribution with a normality test e.g. the Anderson-Darling normality test. If the data were normally distributed, a parametric test was used and if the data were not normally distributed, a non-parametric test was used. From the study design, a paired T-test (Parametric) or a Wilcoxon Signed Rank test (non-Parametric) were used to analyse the data. A probability level equal to or less than 0.05 was chosen to indicate significant differences between number of eggs deposited by gravid females tested in the oviposition choice chamber on the test site (treatments: with number of eggs or extract of eggs) and the control site (treatment: no eggs or hexane).

For the Y-tube bioassays result analysis, one proportion exact test was used to determine whether a greater proportion of females was attracted to each test treatment (5 or 30 males) or control (blank) than would be expected by chance (50/50). The probability level equal to or less than 0.05 was chosen to indicate significant different between test arm and control arm.

Statistical analyses applied for courtship behaviour were paired T-tests or Wilcoxon Signed Rank tests (whichever was more appropriate depending on the distribution of the data) for comparison of courtship behaviour between males and females, and also Fisher's Exact test for predicting the most likely and unlikely behaviour that would be displayed in the mating success.

CHAPTER 3: OVIPOSITION RESPONSE OF GRAVID *PHLEBOTOMUS ARGENTIPES* TO CONSPECIFIC EGGS AND SOLVENT EXTRACT OF CONSPECIFIC EGGS.

3.1 INTRODUCTION

Oviposition attractants and/or stimulants on conspecific eggs or even larvae or pupae of many species of haematophagous insects such as mosquitoes, blackflies and phlebotomine sand flies have been demonstrated by many workers. Oviposition pheromones when combined with kairomones for example environmental cues, may help gravid females to locate suitable sites for oviposition, which is crucial to maximise the survival of their progeny. It has been established that a suitable oviposition site is normally one where ample food and an appropriate environment are provided for the immature stages of insect vector. A gravid female therefore must identify the best (most suitable site) from many competing options and using cues from the eggs laid by other conspecific females may be a part of the multitude of infochemicals that contribute to a decision (McCall and Cameron, 1995).

One of the clearest examples of an oviposition pheromone is from *Culex* mosquitoes. Osgood (1971) showed that gravid *Culex tarsalis* (Say) females preferred to lay their egg rafts in water containing conspecific eggs compared to

distilled water. Similarly gravid *Cx. quinquefasciatus* choose sites that have eggs of other females present. The oviposition pheromone has been identified as erythro-6-acetoxy-5-hexadecanolide. It is found in the apical droplets of their egg rafts, is the main volatile pheromone component (Laurence and Pickett, 1982; Laurence *et al*., 1985). This chemical compound is responsible for attracting gravid females to lay their eggs around previously laid egg rafts (Laurence and Pickett, 1985). In *Aedes aegypti*, fatty acids of chain length C16 to C18 and their methyl esters are the major compounds in the egg cuticular lipid extract (Ganesan *et al*., 2006). They also showed that dodecanoic and (Z)-9-hexadecanoic acid are oviposition attractants. They showed that a greater numbers of eggs were laid on the water treated with either one of the compounds obtained from conspecific eggs compared to the distilled water control. The numbers of eggs laid were comparatively increased when the concentrations of those compounds were increased (1, 10 and 100 ppm). Conversely, methyl esters were shown to deter oviposition at a higher concentration (more than 1 ppm). In these experiments, more eggs were laid in the 'control' water compared to 'treated' water (Ganesan *et al*., 2006).

Oviposition traps are used to collect eggs and/or gravid females for monitoring vector mosquito populations e.g. *Ae. aegypti* and *Ae. albopictus* (Mogi *et al*., 1988) and *Culex* mosquitoes (Reiter *et al*., 1986). The efficacy of ovitraps can be increased when combined with oviposition attractants (Ritchie, 1984, 2001; Reiter *et al*., 1991). Ritchie (1984) demonstrated the use of oviposition attractants; a combination of hay infusion with isopropyl alcohol in a baited CDC light trap

proved to be 88% more effective than carbon dioxide baited CDC light traps in collecting gravid *Culex* mosquitoes. Reiter *et al*. (1991) demonstrated that a pair of ovitraps containing a hay infusion and 10% dilution of hay infusion in tap water, yielded an eight-fold greater catch than CDC ovitrap containing tap water only.

Blackflies of the *Simulium damnosum* complex, exhibit preferences in selecting oviposition sites where conspecific eggs are already present (McCall and Cameron, 1995; McCall *et al*. 1997a). McCall *et al*. (1997a) showed species or forms of the *S. damnosum* complex; *S. sanctipauli*, *S. squomosum*, *S. sirbanum* and the Bioko form, have similar chemical components in the oviposition aggregation pheromone. Then, McCall *et al*. (1997b) again demonstrated that egg extract of *S. yahense* and extract of gravid ovaries of *S. leonense* have similar major chemical peaks (named by them as peak A and B) in common and those two peaks were associated with the response of ovipositing gravid females, and these coincided with earlier studies. Thus, they have suggested traps developed using the oviposition pheromone baits might be effective against all of the *S. damnosum* species complex in West Africa.

McCall and Cameron (1995) suggested that the potential use of oviposition pheromone, combined with kairomones (environmentally derived oviposition attractants) would enhance the attractiveness of the traps. These enhanced traps could be used as a tool in monitoring and controlling vectors as they would allow regular sampling to estimate vector population size and structure, and would be specific to the target species. Furthermore, they would also specifically attract

gravid females (which may have been exposed to infection at their previous blood meal). This would have a particular epidemiological significance as the size and distribution of the infected host population could be more accurately assessed. Recently fed haematophagous insects are generally resting while they digest the blood-meal and unfed females have not yet been exposed to the population of infected host animals.

Gravid *Lutzomyia longipalpis* prefer to oviposit more eggs in the presence of conspecific eggs (El Naiem and Ward, 1991; El Naiem *et al*., 1991). El Naiem and Ward (1991) demonstrated in simple laboratory bioassays that the presence of eggs (from 80 to 320) caused gravid females to lay their eggs in the vicinity of the conspecific eggs when compared to no eggs. They found that 160 eggs induced the optimal response from ovipositing females. In the same study, they also showed that eggs that had been washed rigorously with hexane, ethyl acetate and lastly with distilled water, thus removing any potential attractive chemicals, did not attract gravid females. This observation indicated that some active chemical compounds were present on the surface of the eggs and were being removed by the washing process. Furthermore, this demonstrated that the oviposition response was mediated by a chemical cue rather than physical cue. Further studies of *Lu. longipalpis* oviposition pheromone have shown that the accessory glands are the site of production of the pheromone and the eggs were coated as they pass by in the oviduct. The oviposition pheromone for *Lu. longipalpis* was identified as the C12 fatty acid, dodecanoic acid (El Naiem *et al*., 1991; Dougherty *et al*., 1992, 1994; Dougherty and Hamilton, 1997). Studies on oviposition

attractants and stimulants in this species have shown that frass, larval rearing medium, rabbit faeces and rabbit food attracted gravid females and stimulated them to lay their eggs (El Naiem and Ward, 1992b; Dougherty *et al*., 1993). Experiments with gravid *Lu. longipalpis* demonstrated that the texture of the oviposition substrate was also important in inducing oviposition by gravid females thus a rough surface with small scale crevices was more stimulating to ovipositing females than smooth surfaces and they lay more eggs, this is defined as thigmotropic behaviour (El Naiem and Ward, 1992a).

The studies outlined above suggested that gravid females of insect vectors have a positive response towards conspecific eggs (oviposition pheromone) and also environmental cues (kairomones). Together these enhance the attractiveness of the oviposition site and may improve the survival of the progeny. Based on the information available on the presence of oviposition pheromones and kairomones in the New World sand fly species, *Lu. longipalpis* and the presence of an oviposition pheromone in the Old World species, *P. papatasi*, it is reasonable to speculate that an oviposition pheromone may also occur in *P. argentipes*. Therefore, this study aimed to determine if an oviposition pheromone was present on the eggs of *P. argentipes* by observing the numbers of eggs laid on both control and treatment sides. Two sets of experiments were carried out that replicate the work done in other sand fly species. In the first experiment, freshly laid eggs were tested to determine if their presence caused an increase in the number of eggs laid by gravid *P. argentipes*. In the second experiment, eggs were washed with a

solvent to determine if the extract could induce a similar oviposition response from gravid females as fresh eggs.

3.2 MATERIALS AND METHODS

Adult *P. argentipes* females used were from the colony and were maintained as previously described in Chapter 2.

3.2.1 Experimental Conditions

Oviposition experiments with fresh eggs and egg extracts were carried out in a bioassay arena (modified oviposition pot) in the sand fly insectary at 28°C±2°C using gravid female sand flies i.e. they were three days post blood meal. Oviposition experiments lasted for four days. The adult female flies had been blood fed on mice. The work with the mice followed a UK Home Office protocol for feeding and handling and were carried out by an appropriately qualified Home Office license holder. Blood-fed female sand flies were kept for three days in an adult holding cage in the insectary to allow blood-meal digestion and development of eggs before the experiment started.

3.2.2 Gravid Females

One hundred and twenty engorged females were removed three days after blood feeding from the adult holding cage and placed into a new cage. Twenty males were added to ensure that the females were mated. The cage was kept for three days to allow complete oogenesis and defaecation before starting the

experiment. Four replicates with thirty females in each replicate were prepared at a time. In total, there were sixteen replicates for each treatment.

3.2.3 Oviposition Arena

The oviposition arena was a modified standard colony pot (described in Chapter 2). The design of the arena was adapted from the study of El Naiem and Ward (1991). Freshly prepared Plaster of Paris was poured into a Nalgene pot to a depth of approximately 1 cm, to produce a solid base. It was then put in a plastic box and left to dry at room temperature for a day. After it was dry, the surface of the plaster layer was divided into two halves, which were designated test and control sides. Within each half of the pot, an area was marked out using sharp forceps, into eight (1 cm x 1 cm) squares bounded by 3 mm deep groove (Figure 3.1). The number of eggs laid within the ‘eight square’ area were counted and recorded accordingly. Eggs laid within the area marked by the 3 mm grooves and the grooves themselves were counted, eggs laid outside this area were not considered.

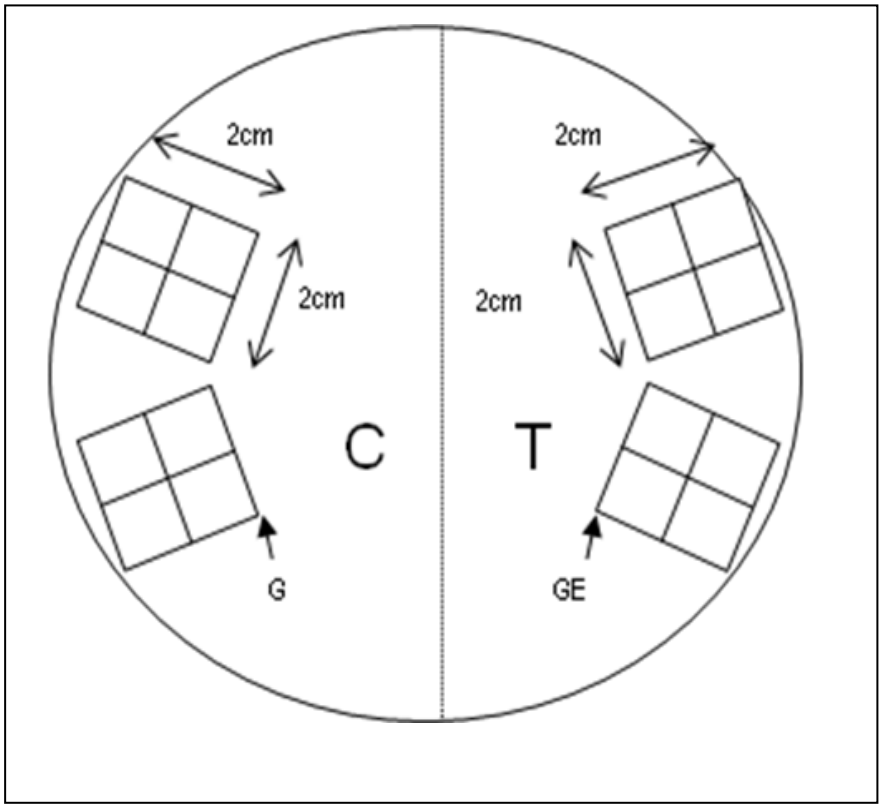

Figure 3.1: A diagram of ovipositional arena of conspecific eggs; T= test and C = control oviposition sides on surface of the Plaster at the bottom of an oviposition arena (11 cm diameter, 7 cm height) used to study the influence of whole eggs on the oviposition of gravid *P. argentipes* females.

G = 3 mm deep groove along the drawn line left empty with no eggs (control side);

GE = 3 mm deep groove in which varying numbers of eggs i.e. 40, 80 and 160 (treatments) placed along the groove (test side).

3.2.4 Eggs

Eggs used for both the “fresh eggs” and “egg extract” experiments were previously laid by colony females. The eggs had been laid in the rearing pots and were 1–2 days old. They were transferred using a fine needle either into the oviposition arenas or into specially prepared clean glass ampoules for preparation of egg extract. The eggs used were carefully handled and counted and ranged in number from forty to three hundred and twenty depending on the experiment.

3.2.5 Preparation of Egg Extract

Glass vials were prepared form Pasteur pipettes as described in Section 2.2.4. After they had cooled to room temperature, a batch of eggs was transferred into the ampoule using fine needle. Ampoules containing 80, 160, 240 or 320 eggs were prepared in this way. The vials were tapped gently so that all of the eggs placed in the vial fell to the bottom and would therefore be covered in solvent. About 10 µl of n-hexane (Pesticide grade residue analysis, BDH Chemicals, UK) which was sufficient to cover the eggs, was then added to the vial. The ampoules were heat–sealed at the open end, labelled with preparation date, number of eggs used and stored at -20°C until use.

3.2.6. Oviposition Experiments

3.2.6.1 *Whole Egg Experiments*

For the whole egg experiments, the plaster of Paris in the bottom of the choice arena was soaked with distilled water for about thirty minutes prior to the introduction of the eggs and then dried with a paper towel. Eggs were then transferred gently with a fine needle into the grooves of the eight (1 cm x 1 cm) squares on the 'test' side at the bottom of oviposition arena equally. The other side (control) was left blank. Experiments with 0, 40, 80, 160, and 320 eggs on the 'test' side were carried out. A control experiment with zero eggs on both sites of the oviposition chamber was used to ensure that there was no positional bias inherent in the experiment.

Nylon netting was used to cover the top of the oviposition arena to prevent gravid sand flies from escaping. Thirty gravid females were transferred with an aspirator into the arena via a small hole blocked with a small piece of cotton wool. Another ball of cotton wool soaked with 60% glucose solution was put on top of the netting to provide the flies with nutrition. A group of four oviposition arenas was prepared for each different number of eggs used at one time. Oviposition arenas were labelled with the date that the sand flies were blood fed. The position of the 'test' and 'control' side were clearly marked. The oviposition arenas were then placed individually in a 2.4 l plastic box (13 cm x 13 cm x 14 cm) along with dampened paper towels on the bottom of the box and a lid fitted. All boxes

containing the oviposition arenas were then placed in a 49 l black box (30 cm x 38 cm x 43 cm) to ensure total darkness for oviposition.

The position of the individual oviposition arenas was rotated randomly to avoid positional bias every day for three days. After four days, all the gravid females (dead or alive) were removed from the chambers. The numbers of eggs laid within the ‘test’ and ‘control’ were noted and recorded separately for each treatment.

3.2.6.2 *Egg Extract Experiments*

The oviposition arena was prepared as previously described in 3.2.6. A piece of triangular filter paper (½ mm x 10 mm x 13 mm) was placed on both the test and control side of the oviposition arena between the eight (1 cm x 1 cm) squares (Figure 3.2). Extract of 80, 160, 240 and 320 eggs were used and sixteen replicates were carried out for each treatment. The vials containing the extracts were snapped at the top and the 10 µl aliquot of hexane containing the egg extract was removed from the vial with a small (10 µl) syringe and placed onto the triangular piece of filter paper on the ‘test’ site, while 10 µl of n-hexane was placed with a separate syringe onto the filter paper on the ‘control’ site. The rest of the experiments were carried out as for the experiment with whole eggs (3.2.6.1). After four days, dead and alive females were removed from the arena, and the numbers of eggs laid on both test and control sides, were counted and recorded accordingly.

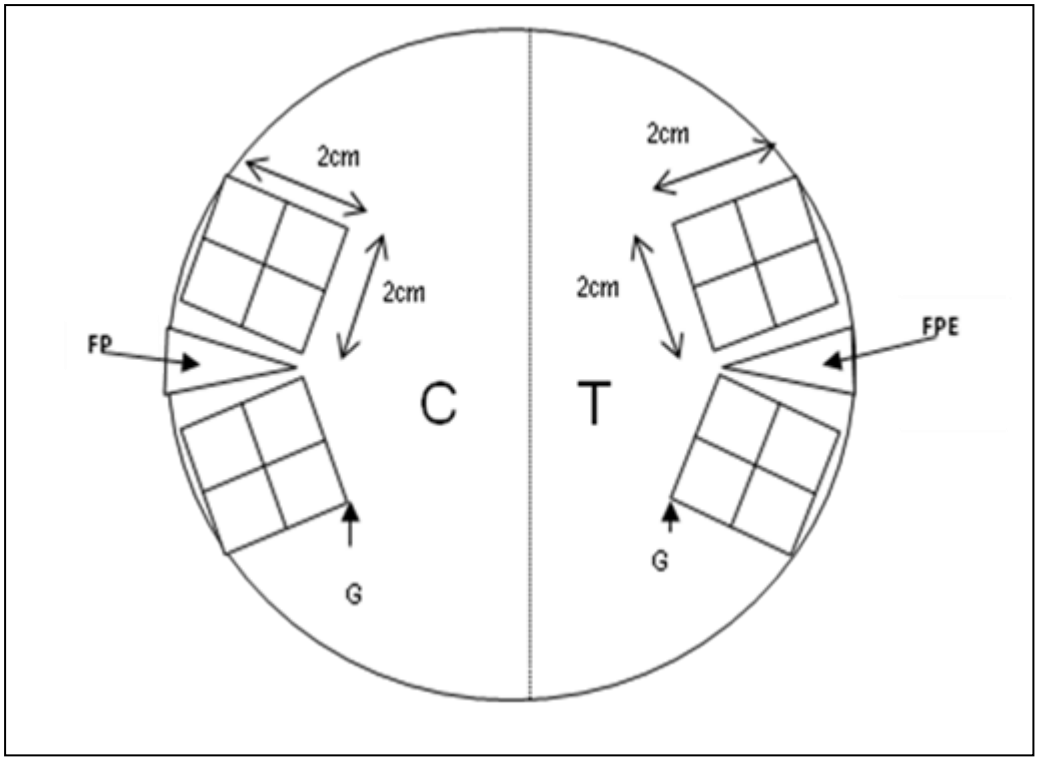

Figure 3.2: A diagram of ovipositional arena of conspecific eggs extract; T = test and C = control oviposition sides on the surface of the Plaster at the bottom of an oviposition arena (11 cm diameter, 7 cm height) used to study the influence of extract of eggs on oviposition of gravid *P. argentipes* females.

G = 3 mm deep groove along the drawn line; numbers of eggs laid were counted within the area of 8 (1 cm x 1 cm) squares

FP = Filter paper containing only hexane (control); Triangular filter paper (½ mm x 10 mm x 13 mm)

FPE = Filter paper containing hexane extract of conspecific eggs (test); Triangular filter paper (½ x 10 mm x 13 mm)

3.2.7 Data Analysis

Data recorded from each experiment were compiled and kept in a Microsoft Excel file. Descriptive statistics including percentages, means and standard error of means were calculated. Whilst for the inferential statistics, data were analysed using Minitab 16. In the first experiment a two sample paired t-test (two-tailed test) was used to determine if differences in the mean numbers of eggs laid on the test or control side were significantly different from each other. A one-way ANOVA was used to analyse data obtained from the second experiment. Probability levels equal to or less than 0.05 indicated a significant different in the mean number of eggs laid.

3.3 RESULTS

3.3.1 Oviposition Response of Gravid *P. argentipes* to Different Numbers of Pre-existing Eggs

The oviposition response of gravid *P. argentipes* females towards different numbers of eggs present on the 'test' side is shown in Table 3.1. When 80 or 160 eggs were placed on the test side, a significantly greater number of eggs were added to this side by the females, compared to the control (80 eggs: Paired t-test, t=2.36, *P*=0.032; 160 eggs: Paired t-test, t=4.35, *P*=0.001). Although the response to forty eggs was not significant, the trend was towards significance (40 eggs: t=1.99, *P*=0.065). The number of new eggs laid in the presence of 320 eggs was greater than the control but the difference was not significant (320 eggs: t= 0.43, *P*=0.336). These results indicate that gravid females were more likely to oviposit in the vicinity of eggs that had already been laid but within a relatively narrow range of numbers of eggs. The control experiments (Table 3.1) with no eggs on either the test or control sides showed that there was no significant difference in the means number of eggs laid on either sides: t=1.05, *P*=0.311) indicating that the apparatus was unbiased.

3.3.2 Oviposition Response of Gravid *P. argentipes* to Hexane Extracts of Different Numbers of *P. argentipes* Eggs

A preliminary experiment showed that the oviposition response of gravid female *P. argentipes* to hexane extracts made by placing eggs in hexane for

differing lengths of time was variable. Although it was not possible to discern a pattern the experiment was standardised by using extract made from eggs that were kept for two hours in hexane. The summary data is presented in Table 3.2.

Only hexane extract made from two hundred and forty eggs elicited a significant oviposition response (Paired t-test, t=3.17, *P*=0.006). Extracts made from the other egg batches did not elicit a significant oviposition response (Table 3.3).

The control experiments with hexane only on the test and control sides (Table 3.3) showed that there was no significant difference in the means number of eggs laid (t=1.10, *P*=0.290) indicating that the apparatus was unbiased.

Table 3.1: The mean number of eggs (± SE) laid by 30 gravid females *P. argentipes* on the test and control sides of the oviposition arena in response to the different numbers of eggs (0, 40, 80, 160 and 320) placed on the test side.

Treatments (No. of eggs placed on the test site)	**Eggs laid by gravid females (mean ± SE)**		***P***
	Test	Control	
CONTROL (No eggs)	431.4 ± 38.0	378.3 ± 22.7	0.290
40	431.9 ± 20.3	370.0 ± 18.1	0.065
80	510.2 ± 28.5	393.8 ± 28.5	**0.032***
160	535.0 ± 22.0	424.6 ± 19.7	**0.001****
320	433.7 ± 44.1	407.9 ± 41.9	0.336

N = 16 for each treatment; Probability level of paired t-test is at 95%; *significant at $P < 0.05$; **significant at $P < 0.01$

Table 3.2: The mean number of eggs (±SE) laid by 30 gravid females *P. argentipes* on the test sides of the oviposition arena in response to extract made from different numbers of eggs (80, 160) for different lengths of time (0.17, 2, 24 hrs) placed on the test sides.

Treatments		**Eggs laid by gravid females (Mean ± SEM)**	***P***
(No. of eggs extracted with hexane on the filter paper at the test side)	*Length of time eggs kept in hexane (hours)*	*Test side*	
80	0.17	352.9 ± 17.5	
	2	485.3 ± 35.2	**0.003****
	24	375.2 ± 26.3	
160	0.17	412.9 ± 30.6	
	2	284.1 ± 19.5	**0.002****
	24	315.0 ± 22.7	

N=16 for each treatment; Probability level of One-way ANOVA is at 95%; *significant at $P < 0.05$; ** significant at $P < 0.01$

Table 3.3: The mean number of eggs (±SE) laid by 30 gravid females *P. argentipes* on the test and control sides of the oviposition arena in response to extract made from different numbers of eggs (80, 160, 240, 320) placed on the test sides.

Treatments (No. of eggs extracted with hexane on the filter paper at the test site)	**Eggs laid by gravid females (Mean ± SE)**		***P***
	Test	*Control*	
CONTROL (No eggs extract)	455.1 ± 33.6	410.6 ± 33.0	0.311
80	485.3 ± 35.2	470.2 ± 33.6	0.787
160	504.6 ± 31.9	514.1 ± 31.7	0.835
240	413.9 ± 27.9	350.8 ± 22.7	**0.006***
320	488.2 ± 27.8	516.3 ± 22.5	0.775

N = 16 for each treatment; Probability level of Paired t- test is at 95%; **significant at $P < 0.01$;

3.4 DISCUSSION

As far as I am aware the results presented here are the first to suggest that *Phlebotomus argentipes* females produce an oviposition pheromone. Oviposition pheromones have been found in other species of sand flies e.g. *Lutzomyia longipalpis* (Dougherty and Hamilton, 1997; Dougherty *et al*., 1992, 1994) and *P. papatasi* (Srinivasan *et al*., 1995). It appears that the pheromone is present on the surface of the eggs and can be removed by washing with an organic solvent and transferred to an alternative surface.

In these experiments, *P. argentipes* females laid more eggs in the vicinity of conspecific eggs when compared to the adjacent blank control side. This effect was observed within a narrow range of numbers of eggs presented on the test side (80 and 160 eggs). When 40 eggs or 320 eggs were placed on the test side gravid females did not increase the number of eggs laid on that side. Overall the effect was weak, on average the percentage increase when the test side was treated with 80 and a 160 eggs, was 12% and 19% respectively by comparison the average increase for *P. papatasi* was approximately 40% (Srinivasan *et al*., 1995). For *Lu. longipalpis*, the average percentage oviposition rate for the test side treated with 80 and 160 eggs was about 68% and 82% respectively.

The results of this study were broadly consistent with the study carried out by El Naiem and Ward (1991), which showed that gravid *Lu. longipalpis* increased

the number of eggs laid in response to conspecific eggs range between 80 to 320 eggs. The results are also in line with those of Srinivasan *et al*. (1995), who found that more eggs were laid in the vicinity of conspecific eggs when 100 or 200 were already present. Srinivasan suggested that the response of gravid *P. papatasi* females was mediated by the chemicals of the surface of the eggs but not by physical (tactile or visual) cues.

Studies both by El Naiem and Ward (1991) and Srinivasan *et al*. (1995) showed that gravid females did not prefer to lay their eggs near washed eggs.

The methods used in this study differed slightly from work of El Naiem and Ward (1991) in their study with *Lu. longipalpis.* Although the oviposition choice chamber design, the condition of the experiments (temperature, humidity, complete darkness) and the number of eggs used in these experiments were the same as for El Naiem and Ward (1991) and Srinivasan *et al*. (1995), different numbers of gravid females were used in both studies. In El Naiem and Ward (1991) and Srinivasan *et al*. (1995), ten gravid females were used whereas in this study, thirty gravid females were used. A preliminary test of numbers of gravid females (ten, twenty, thirty and forty) used in the oviposition experiments showed that ten gravid females did not give as good a response as thirty gravid females (two replicates of each treatment i.e. 10, 20, 30 and 40, data not presented). When low numbers of gravid *P. argentipes* were used, they refused to lay their eggs

either on the ‘test’ or ‘control’ sides suggesting that a threshold concentration of pheromone is an important trigger for oviposition.

The results showed that a significantly greater number of eggs were laid by female *P. argentipes* when extract from 240 eggs was present. This supports the contention that an oviposition pheromone is present on the eggs. However the limited concentration range suggests that other stimuli may be important or that making the extract in hexane or indeed the processing of the extract may have an effect on the outcome. These possibilities need further investigation. This corresponded with the study of an oviposition pheromone of the *Lu. longipalpis* eggs extract (El Naiem and Ward, 1991; Dougherty *et al*., 1992, 1994) and *P. papatasi* eggs extract (Srinivasan *et al*., 1995). The results with only 240 eggs extract showed a significant difference in the response of females and this also supported the earlier experiment with whole eggs, in which there was a limit to the attractiveness of the pheromone.

Compared to other studies of egg extracts of other sand flies, there are similarities and differences in the length of time eggs were kept in solvent, the type of solvent used, the volume of container used, and the number of eggs being used for the extraction. The range of time used to keep the eggs in the solvent was from half an hour in the *Lu. longipalpis* study by El Naiem *et al*. (1991), two hours in the *P. papatasi* study by Srinivasan *et al*. (1995) to twenty four hours in the *Lu. longipalpis* study by Dougherty *et al*. (1992). Varying the length of time that the

eggs were kept in a solvent would have an effect on the types of chemicals and amounts of chemicals extracted from the eggs. The numbers of eggs being extracted were also different; El Naiem *et al*. (1991) used 160 eggs, possibly because it elicited an optimal response from the gravid females of *Lu. longipalpis*, whereas Dougherty *et al*. (1992) used 100 eggs (within the range of eggs that elicited a significant number of additional eggs to be laid on the test side in El Naiem and Ward (1991) i.e. more than 80 eggs). Srinivasan *et al*. (1995) did not describe precisely the number of eggs but extracted 200 eggs of *P. papatasi* in the study. Both El Naiem *et al*. (1991) and Dougherty *et al*. (1992) used hexane as the solvent, but this is a non-polar solvent compared to diethyl ether, which had been used by Srinivasan *et al*. (1995). Compared to my study, the number of gravid females used by previous studies was lower than thirty females. That may be the reason for the higher number of eggs laid in my experiments compared to others. The volume of the container used by El Naeim *et al*. (1991) and Dougherty *et al*. (1992) was 500 ml and this was the same as in my experiment, but Srinivasan *et al*. (1995) used a 250 ml container. Different volumes of the container might affect in the concentration of pheromone or odour released throughout the whole area. Though there were the differences in the studies, all of the findings showed there was an oviposition pheromone associated with the eggs that attracts or/and stimulates ovipositing females sand flies.

From the laboratory observations throughout my research in colony rearing of sand flies, I found that eggs of *P. argentipes* were either clumped or laid singly in a group and were usually found in small numbers compared to *P. papatasi*

(Ifhem Chelbi, personal communication), which were usually clumped in a bigger number, whereas the *Lu. longipalpis* were usually laid singly in a bigger group.

I found clumped eggs of *P. argentipes* in the colony were usually deposited in the groove, whilst singly laid eggs were deposited on the flat surface of plaster of Paris and also in the groove of the rearing pots. The deposition of clumped eggs in the groove by gravid females may be due to the nature of *P. argentipes* oviposition behaviour that is affected by oviposition pheromone and environmental kairomones help to locate suitable places to lay eggs and also their thigmotropic behaviour. The deposition of singly laid eggs may be due to a behaviour whereby the female finds the most suitable place which is safe for their progeny when there is no oviposition pheromone or kairomones that can help them to identify a suitable place to lay their eggs. Unlike in the oviposition choice chambers, I found that 95% of the eggs were laid in the vicinity of the test sites (eggs and egg extract experiments) and the control sites (blank or hexane solvent) support the contention that a pheromone is present. Even though the behaviour of the females was not observed directly, the number of eggs laid support the inference.

Whether the oviposition pheromone of *P. argentipes* is either an attractant or stimulant or both, further experiments should be followed. The experiments carried out in an oviposition arena with the test and control in close proximity give rise to the possibility of contamination and also the possibility that the concentration of pheromone reaches a saturation point quite early thus affecting

the numbers of eggs laid. A different type of bioassay, where the test (with eggs or egg extract) and control (blank) are separated in different containers might help avoid some of these problems. Also the experiments presented here do not make it clear if the chemical present on the eggs is an attractant or a stimulant. Using a Y-tube olfactometer with air passing over the eggs or egg extract in one arm and a blank control on the other arm would allow the gravid females to demonstrate upwind anemotaxis. Further experiments of a similar nature would then be able to clarify if 320 eggs or more were deterrent or repellent or both to gravid females. Additional bioassays using 240 eggs on the test side would be useful to determine if this really does represent an optimal number of eggs. This is an important question because the results seem to suggest that there is an optimal range of numbers of eggs that induce oviposition of gravid females. This would indicate that females will avoid sites that may become overcrowded and there is increased larval competition for scarce resources that would be detrimental to her offspring. Equally if there are very few eggs laid at the oviposition site this may suggest the presence of a predator or other hazard for the offspring.

To support evidence showing that the oviposition pheromone/chemical is associated with eggs or that ovipositing females are attracted to eggs by chemical or physical cues or both, another experiment using washed eggs (washed with hexane) should be pursued. Finally, it would be interesting to see the response of gravid females of *P. argentipes* towards attractants (frass, rabbit food, bacteria, and rabbit faeces) and also to see the combination effects of attractants and

oviposition pheromone whether it would enhance or inhibit the response of the gravid females.

CHAPTER 4: RESPONSE OF VIRGIN FEMALE *PHLEBOTOMUS ARGENTIPES* TO MALE-PRODUCED VOLATILES AND HOST ODOUR.

4.1 INTRODUCTION

Phlebotomus argentipes is the vector of visceral leishmaniasis (VL) in the Indian subcontinent. It occurs in more than 109 districts and causes significant morbidity and mortality amongst the urban and rural poor (WHO, 2006). Although there is some improvement in the treatment options for VL, the drug side effects, availability and cost are still significant problems and as yet no vaccine is available (Croft and Coombs, 2003). Vector control still remains the best way to control the transmission of the disease by reducing contacts between the insect vector and its host. As *P. argentipes* is an anthropophilic vector and Kala-Azar is an anthroponotic disease, reducing contacts between the vector and people offers the best chance for developing new vector control strategies. Part of the solution includes regular surveillance of the insect population densities so that the application of control tools such as insecticides can be optimised.

Integrated vector management including the use of long lasting insecticidal nets (LLIN), indoor residual spraying (IRS) and ecological vector management

(EVM); applying mud/lime mixture to plaster the walls and floors of houses and cattle sheds (Kumar *et al*., 1995) were employed and found successfully to reduce sand fly densities in India (WHO, 1996; Das *et al*., 2008; Kumar *et al*., 2009; Joshi *et al*., 2009). However, continuous use of the chemicals in a vector control programme is not only costly but may also lead to negative impacts on human health and the environment (Alexander and Maroli, 2003; Maroli and Khoury, 2006). Furthermore, the development of insecticide resistance in insect vector populations has been observed in many insect vectors, such as mosquitoes e.g. *Anopheles gambiae* is resistant to DDT, organophosphate (OP) and pyrethroid; *Aedes aegypti* is resistant to pyrethroid, OP and also carbamate; *Culex quinquefasciatus* is resistant to OP and pyrethroid and the sand fly, *P. papatasi* is resistant to DDT in India (Brown, 1986; El-Sayed *et al*., 1989; Chandre *et al*., 1998, 1999; Hemingway and Ranson, 2000).

New methods that are both specific and sensitive are needed to control and monitor the population densities of insect vectors, to help reduce man–vector contact and lead to a reduction of disease transmission. Exploitation of the new methods including genetically modified (GM) insect vector (Crampton *et al*., 1990, 1994), sterile insect technique (Knipling, 1955; Davidson, 1969; Alphey, 2002; Alphey *et al*., 2010), semiochemical based lures (Ritchie, 1984; Morton and Ward, 1990; Reisen and Meyer, 1990; Ward *et al*., 1990; Beehler *et al*., 1994; Millar *et al*., 1994; Kelly and Dye, 1997; Hamilton, 2008; Bray *et al*., 2009, 2010) and others are in progress.

The use of pheromones to attract insects in mass trapping, mating disruption and attracticides (lure-and-kill) strategies have been widely used in agriculture and are an important component of integrated pest management (IPM) programs both for surveillance and control of agricultural pests (Carde and Minks, 1995; Shani, 2000). This new approach could also be adopted for use against insect vectors and the potential exists to exploit the approach in vector control programmes (Oliveira Filho and Melo, 1994; Sonenshine *et al*., 2003; Sonenshine, 2004; Kline, 2006, 2007; El Sayed *et al*., 2009; Bray *et al*., 2010).

Studies of semiochemicals, such as insect-produced pheromones, host and environmental produced kairomones, and their effects on insect vectors have been extensively considered and well documented. For example, in mosquitoes, identification of the oviposition pheromone in *Culex quinquefasciatus* has been established (Laurence and Pickett, 1982, 1985; Laurence *et al*., 1985) and shown to be able to attract gravid females to lay their eggs in the vicinity of their conspecific eggs. Further studies on the chemical compounds surrounding the habitat of the mosquito have also been examined and a few attractants have been discovered that can enhance the efficacy of mosquito traps used in vector control programmes. These include oviposition attractants. For example a combination of hay infusion with isopropyl alcohol used to bait CDC light traps proved to be 88% more effective than carbon dioxide baited CDC light traps in collecting gravid *Culex* mosquitoes (Ritchie, 1984). An evaluation study on the efficacy of a lethal ovitrap (LO) in combination with a kairomone-baited trap (semiochemical attractant) showed that the attractants significantly increased the efficacy of the LO

by attracting more adult mosquitoes, especially gravid females (Ritchie, 2009; Kline, 2006; Rapley *et al*., 2009).

Understanding the behaviour of sand flies in response to their pheromones and kairomones in nature may lead to effective vector control methods that involve the use of pheromone and/or kairomone based lures. *Lutzomyia longipalpis* is an example of a well-studied species especially regarding its sex pheromone and kairomones. In *Lu. longipalpis*, sex pheromone is produced by the male (Lane *et al.*, 1985; Morton and Ward, 1989; Hamilton *et al*., 1994, 2005; Jones and Hamilton, 1998) and emitted from the glandular tissue located in the abdomen (Lane and Ward, 1984). It plays an important role in mating. In lekking behaviour the male emits its' sex pheromone, and in combination with the hosts odour, attracts both males and females to the vicinity of a host animal where both mating and blood-feeding can occur (Quinnell and Dye, 1994). The chemical components of the sex pheromone have been identified (Hamilton *et al*., 1996a, 1996b, 2004, 2005) and the behaviour of female sand flies associated with the pheromone has been studied (Morton and Ward, 1989; Nigam and Ward, 1991) in the laboratory. Research on the effect of host kairomone and pheromone on female *Lu. longipalpis* has demonstrated that host odour synergises the attraction of females to male pheromone (Bray and Hamilton, 2007). A formulation for synthetic pheromone has been developed and its potential for use in a pheromone-based trap, in combination with host presence, has been demonstrated in the laboratory and field (Bray *et al*., 2009, 2010). In Old World sand flies, behavioural evidence

for the presence of a sex pheromone in *P. papatasi* and *P. argentipes* has recently been demonstrated (Chelbi *et al*., 2011; Kumar *et al*., 2012).

The aim of the work discussed in this chapter was to consider behavioural evidence for the presence of sex pheromone in *P. argentipes*. Previous studies have indicated that in a simple behavioural experiment that female *P. argentipes* responded to extract of males (Kumar *et al*., 2012). However the response that was demonstrated was not an anemotaxis which would indicate that the signal was a volatile chemical capable of conveying an attractive message over longer distances. Thus response of female flies to male odour in a moving air bioassay was investigated, the numbers of males required to attract a significant response from females was analysed and extract of males as a source of male odour was examined.

4.2 MATERIALS AND METHODS

Adult males and females were from the colony maintained as previously described in Chapter 2.

4.2.1 Male and Female Sand Flies

Female and male flies used in mating observations were of the same age categories (i.e. 1d (1 day old), 2d, 3d, 4d, 5d and 6d), separated within 5 hours of emergence; before rotation of the external male genitalia; fed only on saturated sugar solution. On the other hand, male and female flies used in the bioassays were 2d and 6d only. Cages of 20 to 50 sand flies were acclimatised to the experimental conditions for 2 hours before starting. The mating success observations were carried out in two different arenas; 1) in the Barraud cage and 2) in the mating arena, while the bioassays were done in a Y-tube olfactometer (Figure 4.1). All of the experiments were carried out on a, vibration-dampened bench in a room used specifically for doing bioassays at 27°C±2°C and 85% relative humidity under white fluorescent light between 0900 and 1300 hours.

4.2.2 Preliminary Studies: Observation of Mating Success and Response in Y-tube Bioassays of Different Age of Males and Females

Two observational studies were carried out to determine the age at which males and females were most likely to mate with each other. Trial bioassays were

carried out to set the parameters for the bioassays in which, unmated males and females aged 1, 2, 3, 4, 5 or 6d were used. The first experiment was carried out in a Barraud cage (20 x 20 x 20 cm) and the second was in an observation chamber, which was a round container (3 cm diameter x 2 cm high) with a 1 cm layer of plaster of Paris at the bottom. The two different mating studies allowed a comparison in the mating response between the environments. Before the observations started, a single male was released into the cage or observation chamber and left for 5 minutes to acclimatise to the setting. Then a female was placed in the chamber and observed for about 30 minutes or until mating i.e. copulation took place. In these observational studies, mating was considered as being successful when the male and female were observed to copulate for more than half a minute. All of the observations were recorded. For every observation, the age of the male and female was the same. The minimum and maximum time taken to achieve of mating success was recorded. Two replicates of ten observations (i.e. 20 pairs) for each of the different age categories (1, 2, 3, 4, 5, and 6d) were completed.

4.2.3 The Y-tube Bioassays

Experiments were carried out to find out whether male flies produced a volatile sex pheromone that could attract females over a relatively short distance in a Y-tube olfactometer. Comparisons of the response of female flies to different numbers of males that could produce sex pheromone in the test side and the effect of different ages were also noted. A Y-tube olfactometer (Figure 4.1) as

used by Hamilton and colleagues (Bray and Hamilton, 2007; Chelbi *et al*., 2011) was used to carry out the bioassays.

The apparatus comprised of a Y-shaped glass tube (Figure 4.2; made from 10mm internal diameter (i.d.) glass tubing), it consisted of a 10 cm long stem that branched into two 10-cm-long arms separated from each other at a 65° angle. Teflon tubing (40 cm x 3 mm i.d.; Supelco Ltd., Gillingham, UK) was attached to both arms by brass Swagelok connectors (Swagelok Company Ltd., Solon, Ohio, USA). The other end of each piece of Teflon tubing was attached to a modified Dreschel head (VWR International Ltd., Lutterworth, UK) inserted into a 50 ml glass round-bottomed flask (one flask was designated as the test [males inside] and the other arm as control arm [empty]). Each Dreschel head was connected to a compressed air cylinder (BOC Gases Ltd., Guildford, United Kingdom) to supply air via Teflon tubing. Airflow was controlled using two-stage regulator (BOC Gases Ltd.) attached to the cylinder and a rotameter to control airflow (Supelco Ltd.). The airflow exiting each flask was adjusted and set at 2.5 ml s^{-1}, resulting in an airflow of 5 ml s^{-1} at the outlet of the olfactometer stem. Airflow was measured with a bubble flow meter. Before passing through the Y-tube olfactometer, compressed air was cleaned by an activated charcoal filter (Supelpure HC, Supelco Ltd.). All connections were then made airtight by sealing with Teflon tape (Sigma Aldrich Company Ltd., Gillingham, UK) and cleaned glass wool was inserted into the inlet end of both arms to prevent flies from escaping into the Teflon connecting tubes. The apparatus was placed horizontally on the vibration-dampened bench during the bioassays.

Different numbers of virgin *P. argentipes* males were placed in the test flask for 30 minutes before the experiment started. A female sand fly was removed from a holding cage using a modified mouth aspirator and placed into the outlet (open end) of the olfactometer stem (Figure 4.1). The movement of the female into the 'test' arm (i.e. the arm connected to the 50 ml bulb holding different numbers of male sand flies) or 'control' arm (blank) was noted when she moved along at least halfway along the length of the chosen arm. If the female made no movement into either of the two arms in a maximum of three minutes she was considered as a non-responder. Female sand flies (either 120 or 150 depending on the experiment) were tested for each treatment over a period of several days. Swapping the position of test and control arms by rotating the Y-tube and connecting tubing through 180^{o} along its longitudinal axis every 10 replicates was done to control any potential effect of a room positional bias. Before each day's trials Teflon tubing was cleaned as previously described. Glass apparatus was cleaned as previously described.

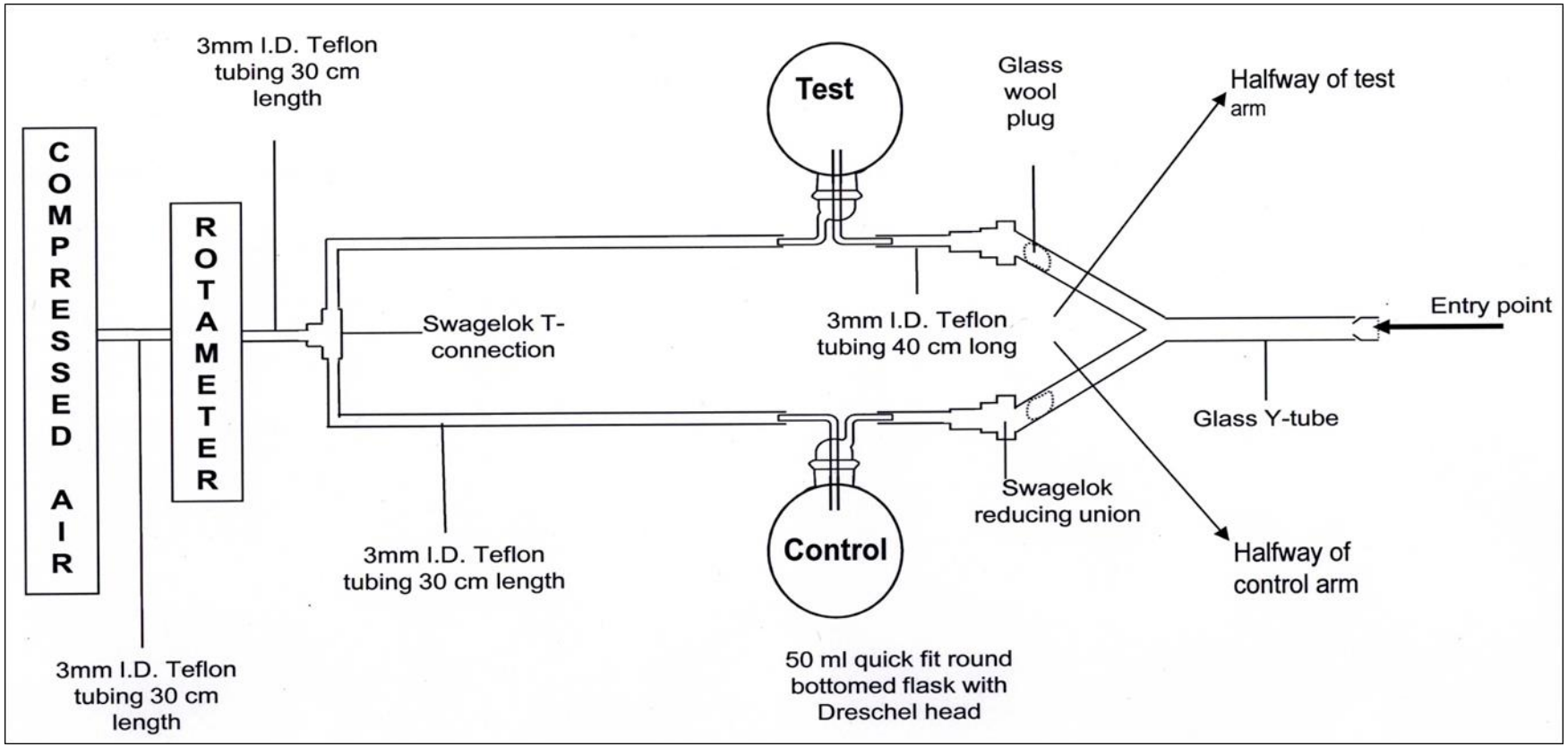

Figure 4.1: Diagram (not to scale) of a Y-tube olfactometer used in the bioassay (host odour not present) (Adapted from Chelbi *et al*., 2010).

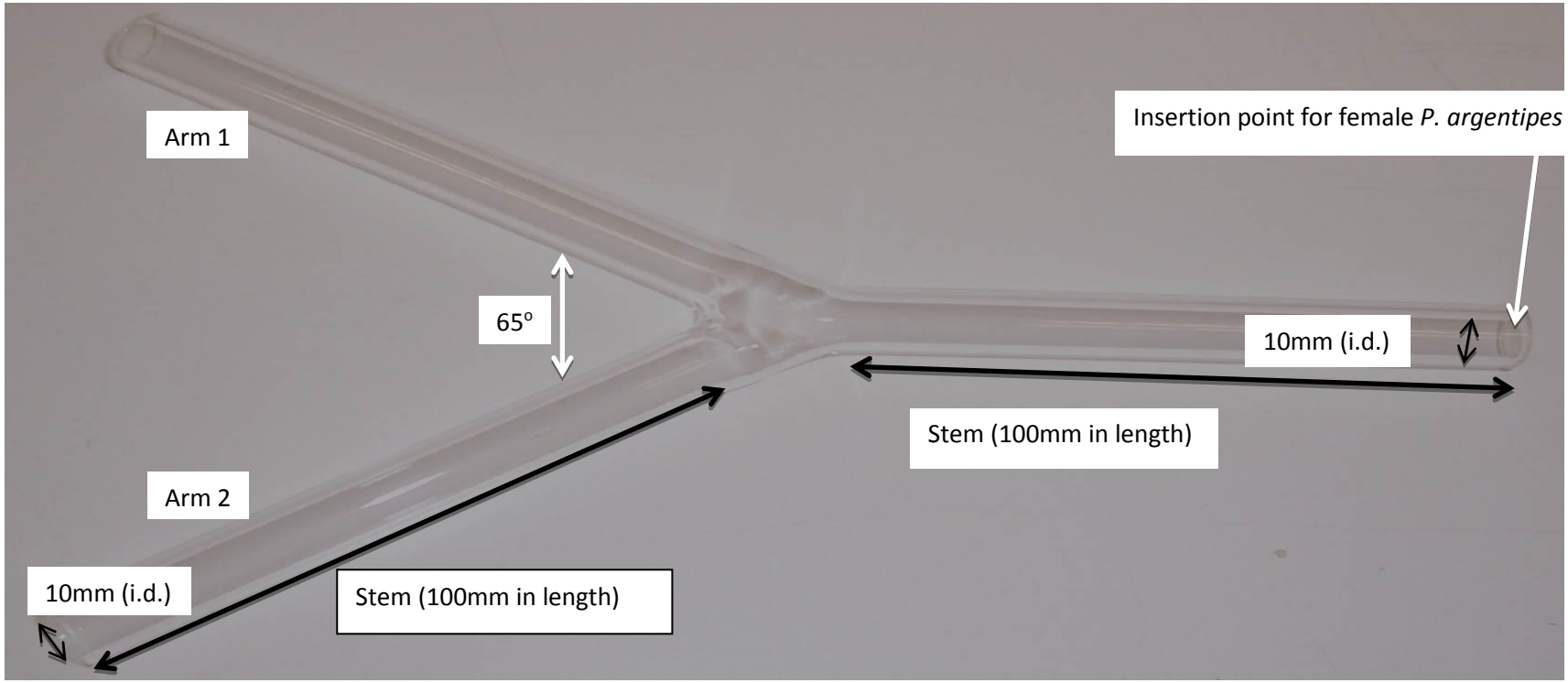

Figure 4.2: A photograph of a Y-tube glass olfactometer showing the insertion point for female *Phlebotomus argentipes* at the end of the stem and the two arms which were designated either test or control. Females were counted as having made a choice when they passed a point halfway along either arm during 3 minutes.

4.2.4 Preliminary Studies: Trial Bioassays without Host Odour

Based on the observational studies of mating success, parameters such as the age of the flies and response times were decided and trial bioassays were performed before addressing the principal aims of the study i.e. to determine if female flies were attracted to male derived odour in the test arm compared to a blank in the control arm. In these trials, the response of male and female flies of the same age (2d or 6d) were compared with each other and also the response of young flies (2d) was also compared with older flies (6d). As the bioassay for *P. argentipes* had not been attempted previously, times for female flies to respond were also tested to establish the best length of time to capture the average response of female flies to the males. Response times of 3 min (Bray *et al.,* 2007) and as long as 5 min have been used previously in bioassays with *Lu. longipalpis*.

However, as *P. argentipes* were observed to be very quiet in their behaviour, it was expected that they would react more slowly than other sand fly species. The numbers of replicates performed also ranged from 40 to 150 females for each treatment over a period of a day. A host was not present in any of these trials. The response of the females to either the test or the control arm was recorded. Those females that responded to neither the test arm nor the control arm were also recorded as non-responders.

4.2.5 Bioassays to Determine the Response of Virgin Female *P. argentipes* to Male Headspace Volatiles with and without the Presence of Host Odour

4.2.5.1 *Response of; i) Virgin (2d) Females to Virgin (2d) Males and; ii) Virgin (6d) Females to Virgin (6d) Males*

To determine if any preference for any one arm of the olfactometer occurred, a Y-tube bioassay without host odour was carried out as described above (Figure 4.1). Before the experiment started, the air was allowed to flow for about half an hour to help remove any contaminating volatiles from the apparatus. Females (n=150) were tested in the apparatus over a period of several days. Again to control for positional bias in the room, the Y-tube and connecting tubing were rotated every 10 replicates as previously described. The response of the females to either the test or the control arm was recorded. Those females that responded to neither the test arm nor the control arm were recorded as non-responders.

4.2.5.2 Response of; i) Virgin (2d) Females to Vrgin (2d) Males and; ii) Virgin (6d) Females to Virgin (6d) Males in the Presence of Host Odour

A Y-tube bioassay was carried out to determine if female flies were attracted to male odour in the test arm in the presence of host odour. Two host animals (mice) were also included in the apparatus, which was set up as described in Section 4.2.3. However a 2 l glass jar with a lid holding two mice was added to provide host odour during the experiment. The glass jar was placed between the rotameter and the round-bottomed flask (Figure 4.3). Females could

then respond to either a combination of host odour and male odour (test arm) or the host odour alone (control arm). Before the experiment was started, air was passed through the apparatus for about an hour. Either five or thirty males that had been anaesthetised by cooling at -20°C for about 30 seconds were added to the flask on the test arm of the apparatus and air was passed through for half an hour. A similar flask containing no males was placed in the control side of the apparatus. Then 150 females were tested individually against the males in the test flask or nothing in the control flask over a period of several days. The response of the females to either the test or the control arm was recorded. Those females that responded to neither the test arm nor the control arm were recorded as non-responders. The outcomes of the bioassays when a host was present or absent were compared and analysed to establish the need to have a host present in the bioassay.

4.2.5.3 Response of; i) Virgin (2d) Females to Virgin (6d) Males and; ii) Virgin (6d) Female to Virgin (2d) Males in the Presence of Host Odour

Experiments were conducted to establish the responses of *P. argentipes* females to males that were either younger or older than those used in the Y-tube bioassay, as described above. Sand flies aged 2d were categorised as young and 6d as old. Tests to determine the responses of young female flies to old male flies and old female flies and young male flies were carried out. Host odour was used to enhance the potential response to male odour in the test arm. 120 females were tested individually in each treatment over a period of days.

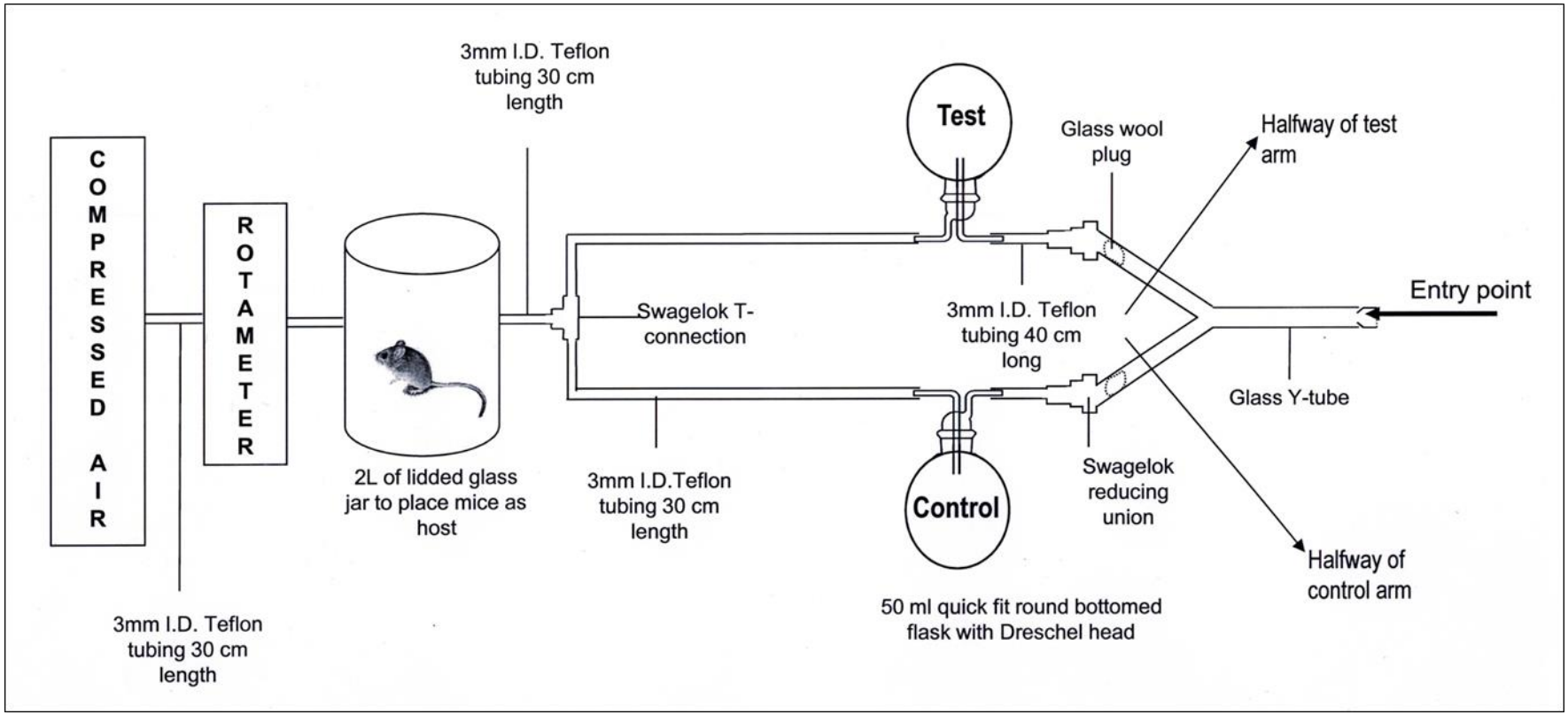

Figure 4.3: Diagram (not to scale) of a Y-tube olfactometer used in the bioassays with host odour present: (Note the additional glass jar used to keep live host animals (mice)).

4.2.6 Preparation of Extract of *P. argentipes* Males

Extract of males were prepared by freezing male flies (6d) in a -4^{o}C freezer for a few minutes to kill them. Dead flies (n=10) were counted and placed in a glass ampoule to which 30 µl of n-hexane (Pesticide Grade Residue Analysis, VWR International Ltd., Lutterworth, UK) was added. Ampoules were prepared from glass vials as described in Chapter 2. The ampoule was sealed by flaming the open end. This gave a final concentration of 0.3 male equivalents (ME) per µl of hexane. The ampoule of male extract was labelled and stored at -20°C until use.

4.2.7 Bioassays of Male *P. argentipes* Extract

A Y-tube bioassay, as described above but with a slight modification was carried out (Figure 4.4). The round-bottomed flasks on both arms were replaced by a shorter piece of Teflon tubing which had a hole (ca. 1 mm diameter) made in it. A piece of filter paper was rolled-up and placed inside this piece of Teflon tubing in each of the test and control sides of the olfactometer. After one hour of allowing host odour to flow through the olfactometer, 3 µl of male extract (=1ME) was injected onto the rolled filter paper on the test arm and 3 µl of hexane was placed on the filter paper in the control arm. After 1 minute, a female was placed into the outlet end of the olfactometer stem to make a choice. All the responses were recorded. Another bioassay to determine any potential positional bias (control bioassay) was carried out without host odour using a Y-tube olfactometer as in Figure 4.5.

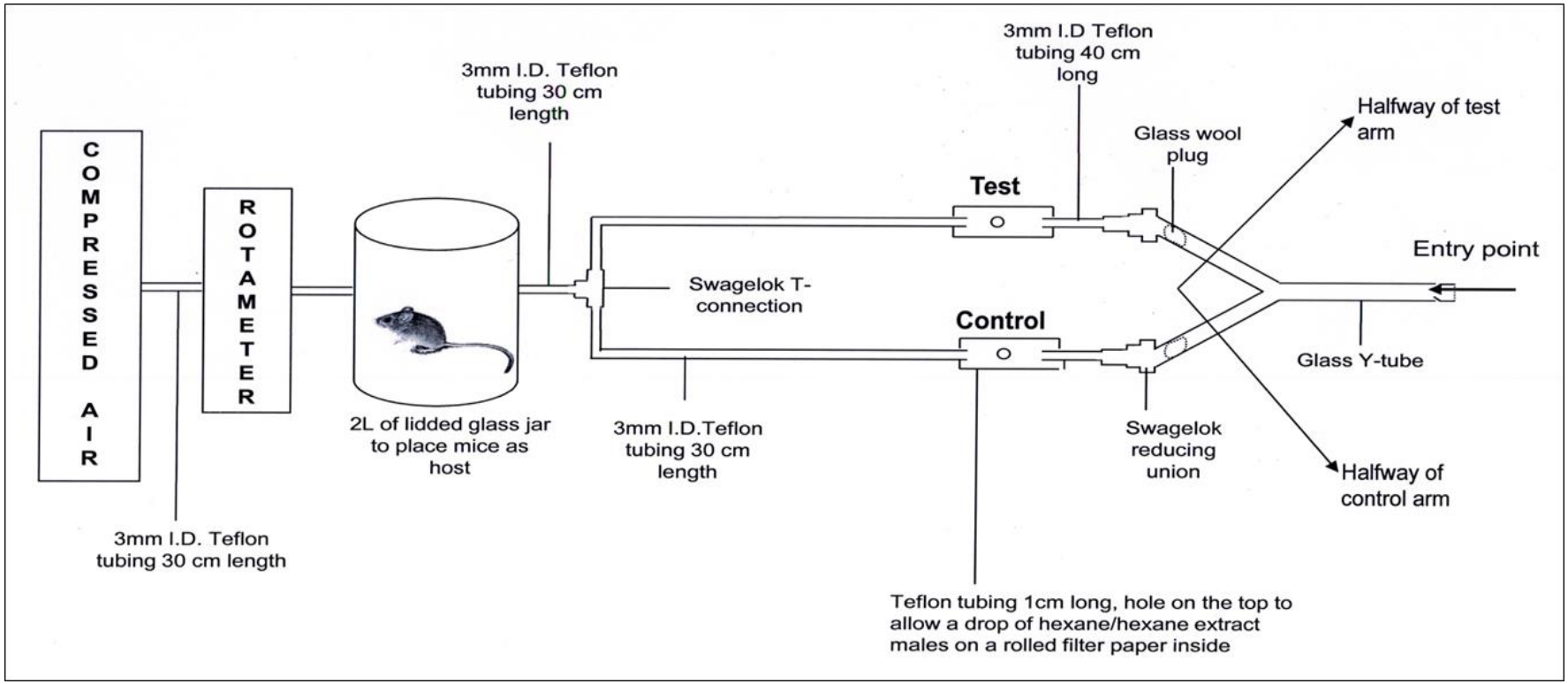

Figure 4.4: Diagram (not to scale) of the Y-tube olfactometer used in the bioassay with male extract. The position of the Teflon inserts, the modification for adding the male extract, instead of the glass bulbs containing live males, can be seen.

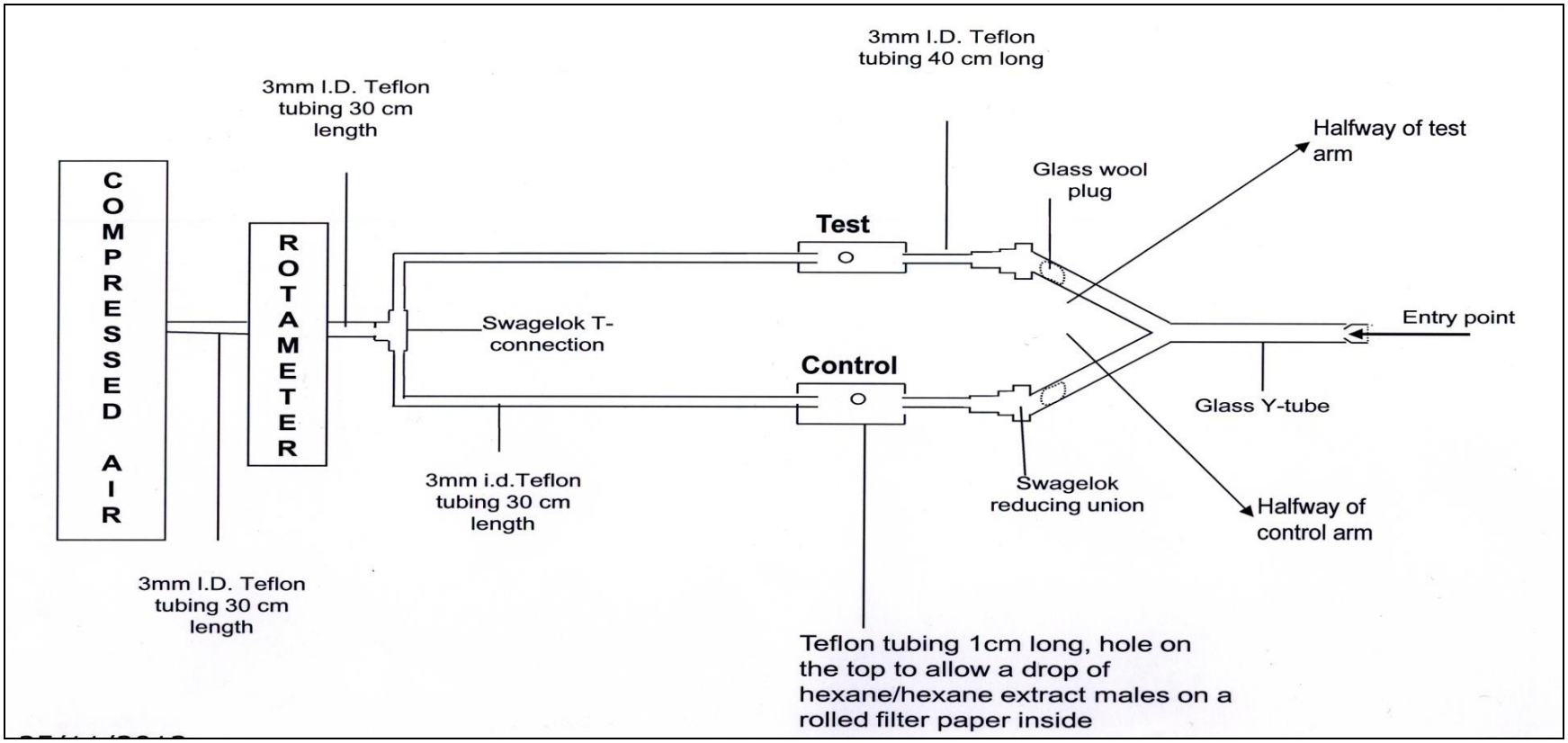

Figure 4.5: Diagram (not to scale) of a Y-tube olfactometer with modification for insertion of extract of males (without host odour).

4.3 RESULTS

4.3.1 Preliminary Study: Observation of Mating Success and Response in Y-tube Bioassays of Different Age Males and Females

For both types of mating arenas 6d *Phlebotomus argentipes* had a higher rate of mating success compared to the other ages (Figure 4.6). The Barraud cage appeared to be a much better environment for mating compared to the mating chamber. However both types of arena showed that as the age increased, the proportion of couples that mated increased. It was also found that the duration of mating ranged from three to sixteen minutes in both types of mating environments. These preliminary observations suggested that it would be best to use 6d sand flies in the Y-tube experiments.

The response of 2d (Figure 4.7) females to 2d males was different from the response of 6d females to 6d males (Figure 4.8). Females (2d) chose the control side (no males) but 6d females preferred the test side (males present).

The percentage of non-responders in all the preliminary Y-tube bioassays was consistently higher than those that responded to either the test and control side. The response of the 6d sand flies (Figure 4.8) showed that similar proportions of female sand flies chose either the test side or the control side. In the experiments with 2d sand flies 17.5% of 40 females chose the test side, 30% chose the control side and 53% did not to respond to either side (Figure 4.7).

Among the 6d sand flies (Figure 4.8), 23.3% of females (n=150) showed a response to 5 males, 22% chose the control side and 54.7% showed no response. When 10 males were used in the test side 30% of the females (n=150) chose the test side, 25.3% chose the control side and 44.7% were not responsive. When 20 males were used in the test side, 23.9% of the females (n=46) chose the test side, 19.6% chose control side and 56.5% did not respond. When 25 males were used in the test side 28.7% of females (n=150) chose the test side, 21.3% chose the control side, and 50% did not respond. In none of the trials was there a significant difference in the numbers of females responding to the test arm compared to the control arm. However, the average proportion of 6d females that responded to 6d males (n=5, 10, 20 and 25) was 29 and this was significantly greater than the proportion of 2d females that responded to 2d males (1-proportion exact test: proportion to 6d males (average)=26.5%:proportion to 2d males=17.5%, *P*=0.036).

These results matched the preliminary behavioural observation experiments and suggested that it would be best to use 6d males and females in further experiments.

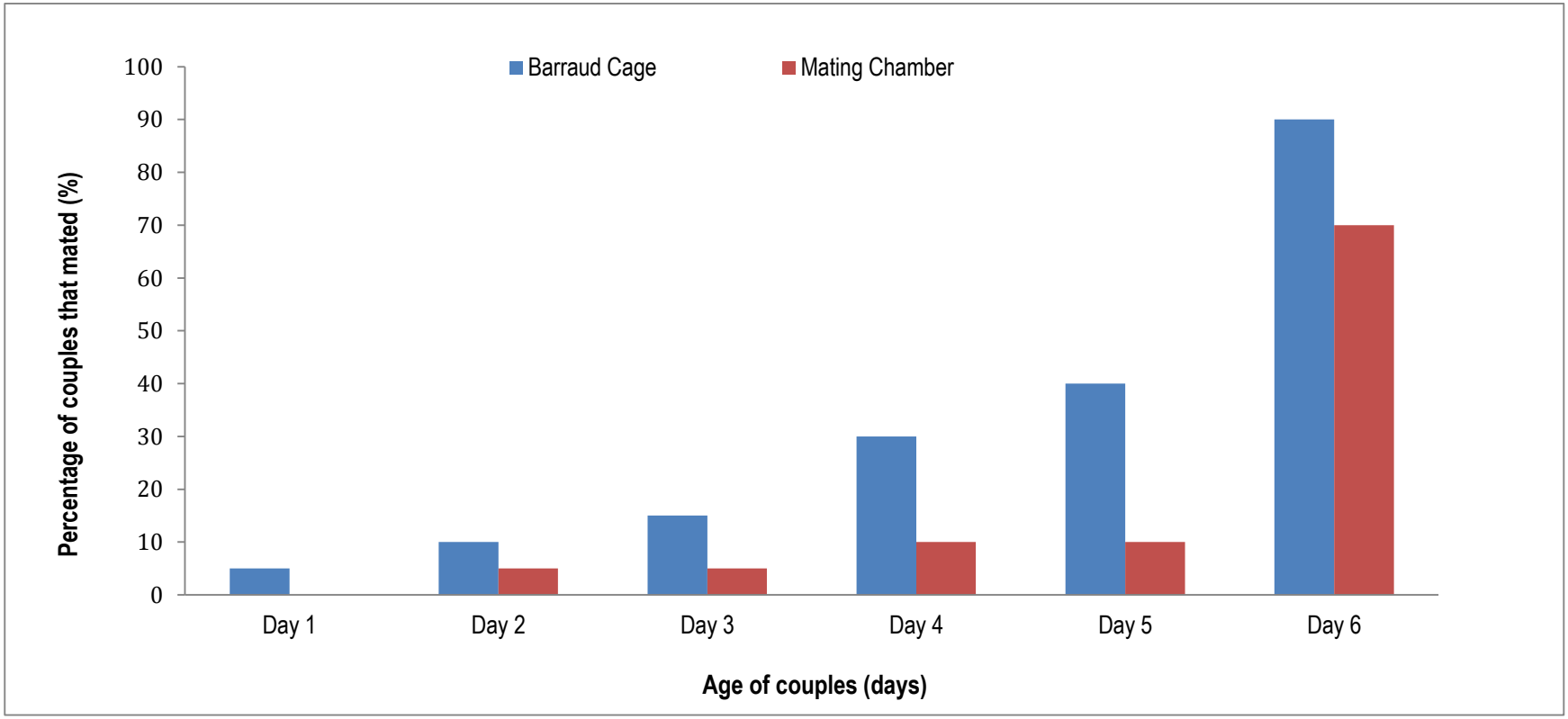

Figure 4.6: Percentage of couples, of different ages (1 to 6d) that successfully mated in two different environments; Barraud cage or mating chamber.

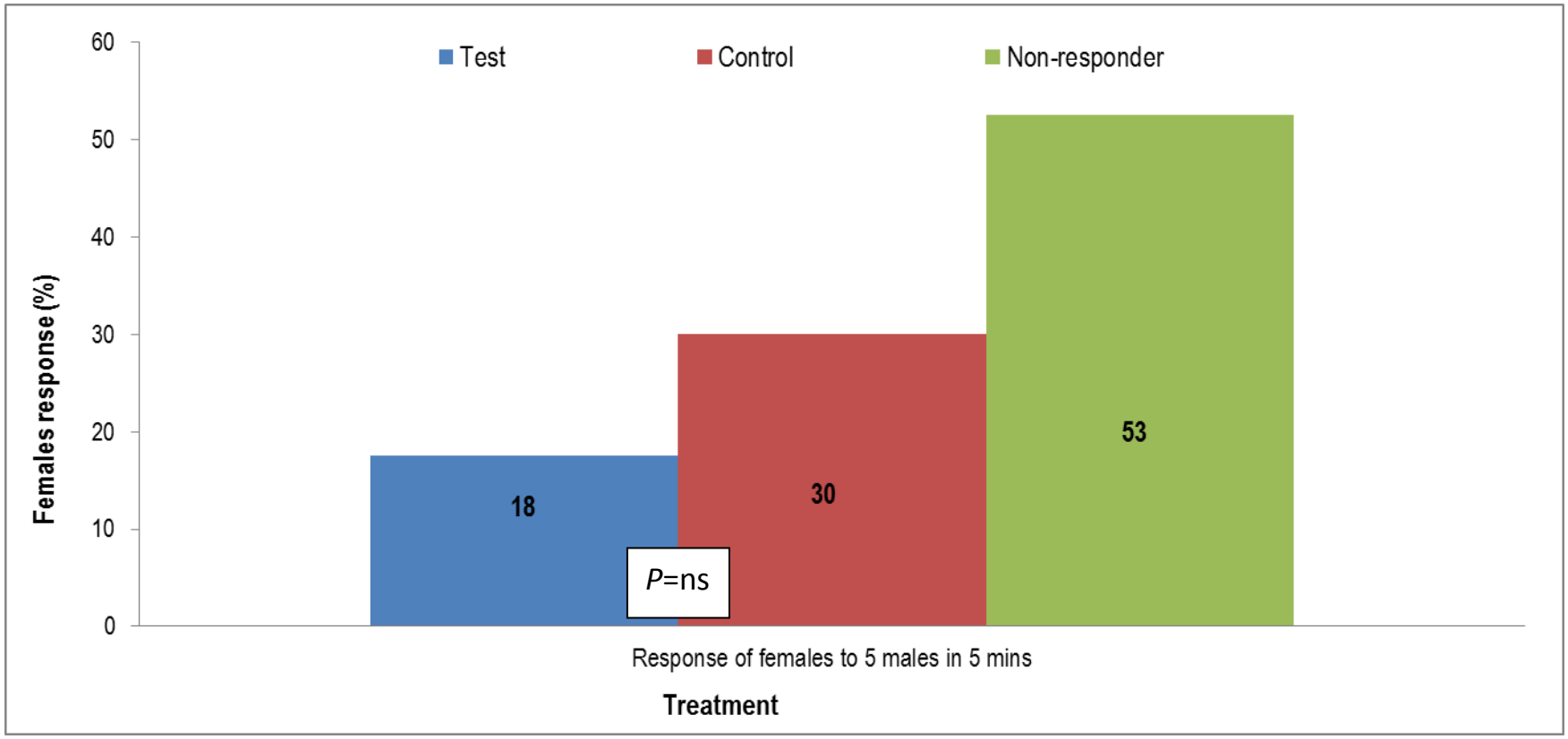

Figure 4.7: Response of *P. argentipes* females (2d) (n=40) to a group of 5 males (2d) in a Y-tube olfactometer during a 5 minutes period in a trial bioassay in the absence of host odour. *P*-value was calculated using a proportional exact test.

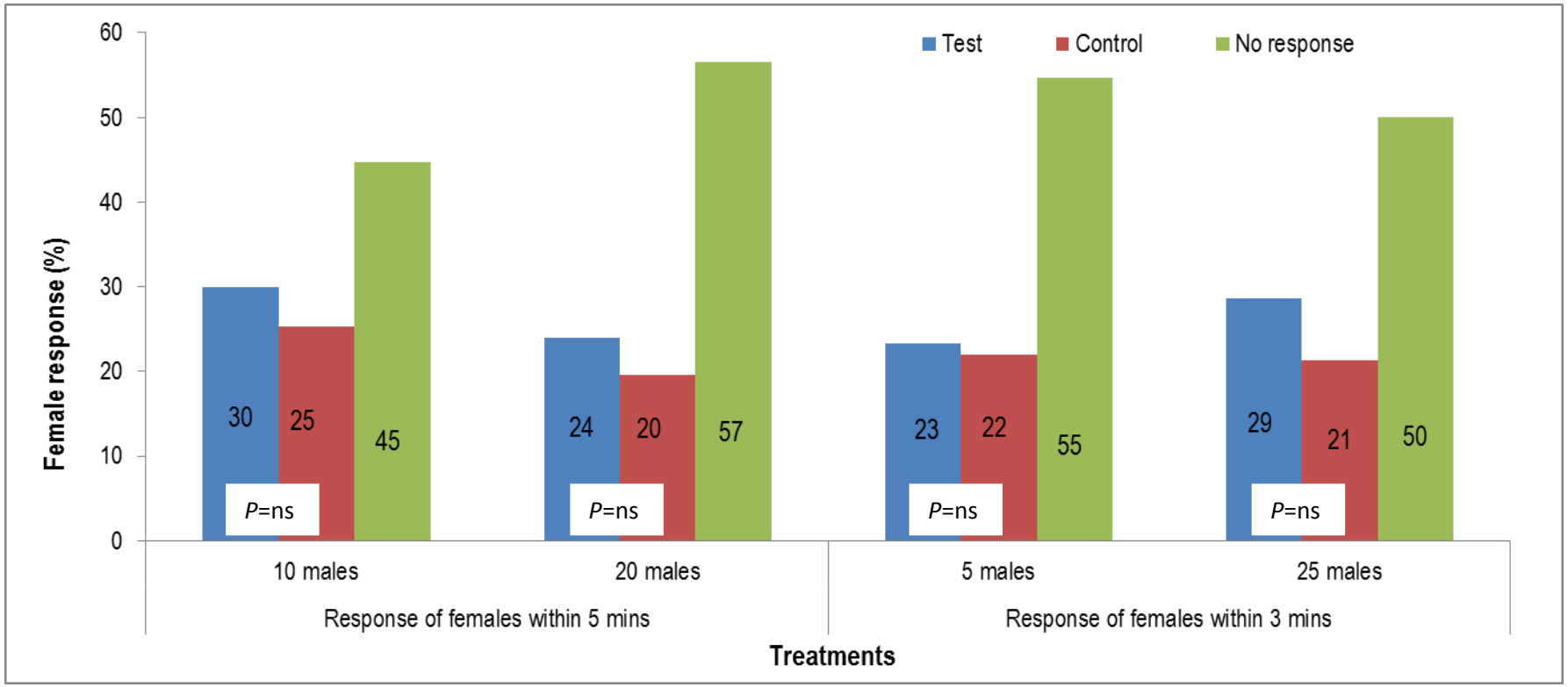

Figure 4.8: Response of female *P. argentipes* (6d) (n=46-150) to different sized groups of males (6d) (n=5, 10, 20 and 25) during 2 time periods (3 or 5 mins) in a Y-tube (host odour was absent). *P*-value was calculated using a proportional exact test.

4.3.2 Response of *P. argentipes* Females (2d) (n=120) to Males (2d) (n=5 and 30) and Females (6d) (n=150) to Males (6d) (n=5 and 30) in a Y-tube Olfactometer in the Presence of Host Odour

Female *P. argentipes* (6d) were significantly attracted to a group of 30 males (6d) (1-proportion exact test; number of females to test=94, number of females to control=51; $P<0.01$) but not a group of 5 males (6d) (Exact test: test=83, control=65; $P=0.162$) (Figure 4.9). The control experiments with host odour but no males present in the test arm of the apparatus showed that there was no significant difference in the response of the females to the test arm compared to the control arm (Exact test; test=71, control=76; $P=0.681$). When no host odour was present in the test arm again there was no significant difference in the response of the females to the test arm compared to the control arm (test=40, control=41; *P*=ns) indicating that the apparatus was unbiased.

An interesting observation is that there was a significantly lower number of non-responding female sand flies when host odour was present in the Y-tube olfactometer compared to when it was absent (Exact test; no host odour=69, host odour=2; $P<0.01$).

Female *P. argentipes* (2d) were not significantly attracted to a group of 30 males (2d) (Exact test: number of females to test=62, number of females to control=51; *P*=ns) or a group of 5 males (2d) (Exact test: test=57, control=57; *P*=ns) (Figure 4.10). The control experiments with host odour but no males present in the test arm of the apparatus showed that there was no significant difference in

the response of the females to the test arm compared to the control arm (Exact test: test=55, control=57, *P*=ns). Again when no host odour was present in the test arm there was no significant difference in the response of the females to the test arm compared to the control arm (test=30, control=29; *P*=ns) indicating that the apparatus was unbiased.

There were a significantly lower number of non-responding female sand flies when host odour was present in the Y-tube olfactometer compared to when it was absent (Exact test: no host odour=51, host odour=8; $P<0.01$).

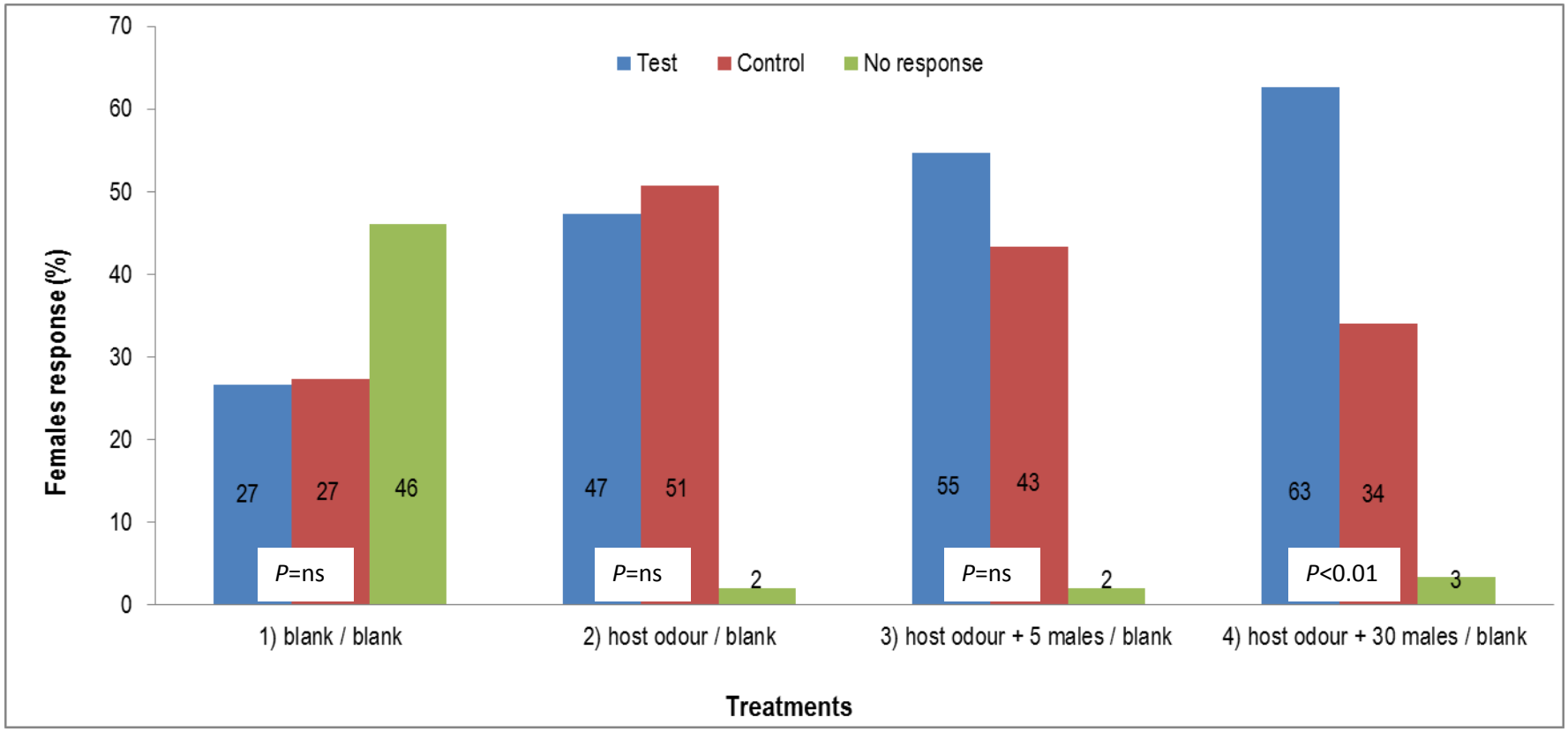

Figure 4.9: Percentage response of female *P. argentipes* (6d) (n=120) in a Y-tube olfactometer to: 1) blank/blank (test/control); 2) host odour/blank; 3) host odour + males (n=5) (6d)/blank and; 4) host odour + males (n=30) (6d)/blank. *P*-value was calculated using a proportional exact test.

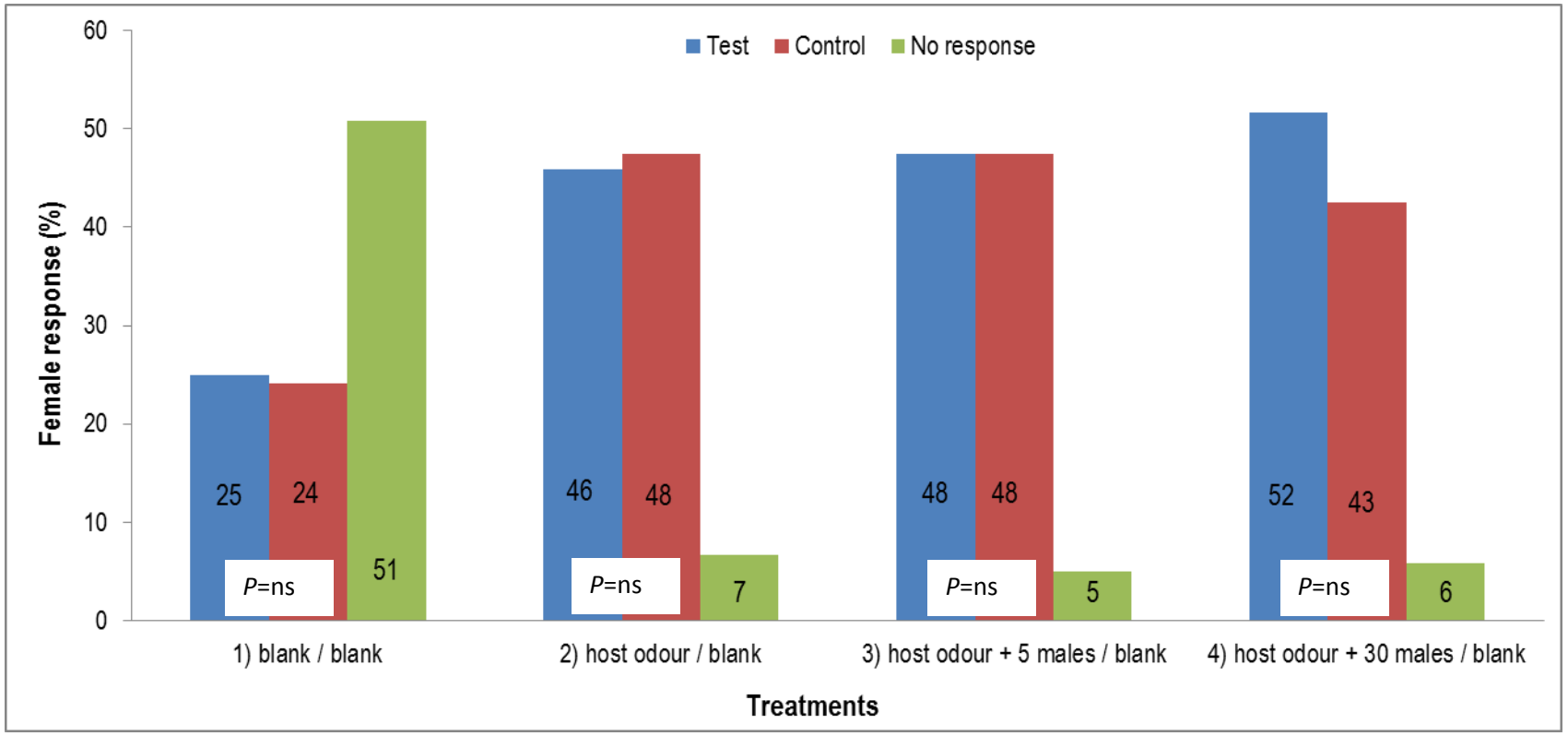

Figure 4.10: Percentage response of female *P. argentipes* (2d) (n=120) in a Y-tube olfactometer to: 1) blank/blank (test/control), 2) host odour /blank, 3) males (n=5) (2d) + host odour/blank and 4) males (n=30) (2d) + host odour/blank. *P*-value was calculated using a proportional exact test.

4.3.3 Response of *P. argentipes* Females (6d) (n=120) to Males (2d) (n=5 and 30) and Females (2d) (n=120) to Males (6d) (n=5 and 30) in a Y-tube Olfactometer in the Presence of Host Odour

Females (6d) were significantly attracted to a small group (n=5) of males (2d) in the presence of host odour (Figure 4.11) (1-Proportion exact test: number of females to test=71, number of females to control=44, *P*=0.015).

However females (6d) were not significantly attracted to a large group (n= 30) of males (2d) in the presence of host odour (Figure 4.12) (exact test; test=60, control=54, *P*=ns).

Females (2d) were not significantly attracted to a small group (n=5) of males (6d) (Figure 4.12) (exact test; test=60, control=47, *P*=ns) and females (2d) were not significantly attracted to a large group (n=30) (Figure 4.13) in the presence of host odour (exact test; test=56, control=52; *P*=ns) and test arm=0.56, control arm=0.44, *P*=ns).

Although the proportion of non-responding females increased from 4.5%, when using 6d females, to 10.5% when using 2d females, this was not a significant increase (exact test; average number of 2d females not responding=13, average number of 6d females not responding=5; *P*=ns).

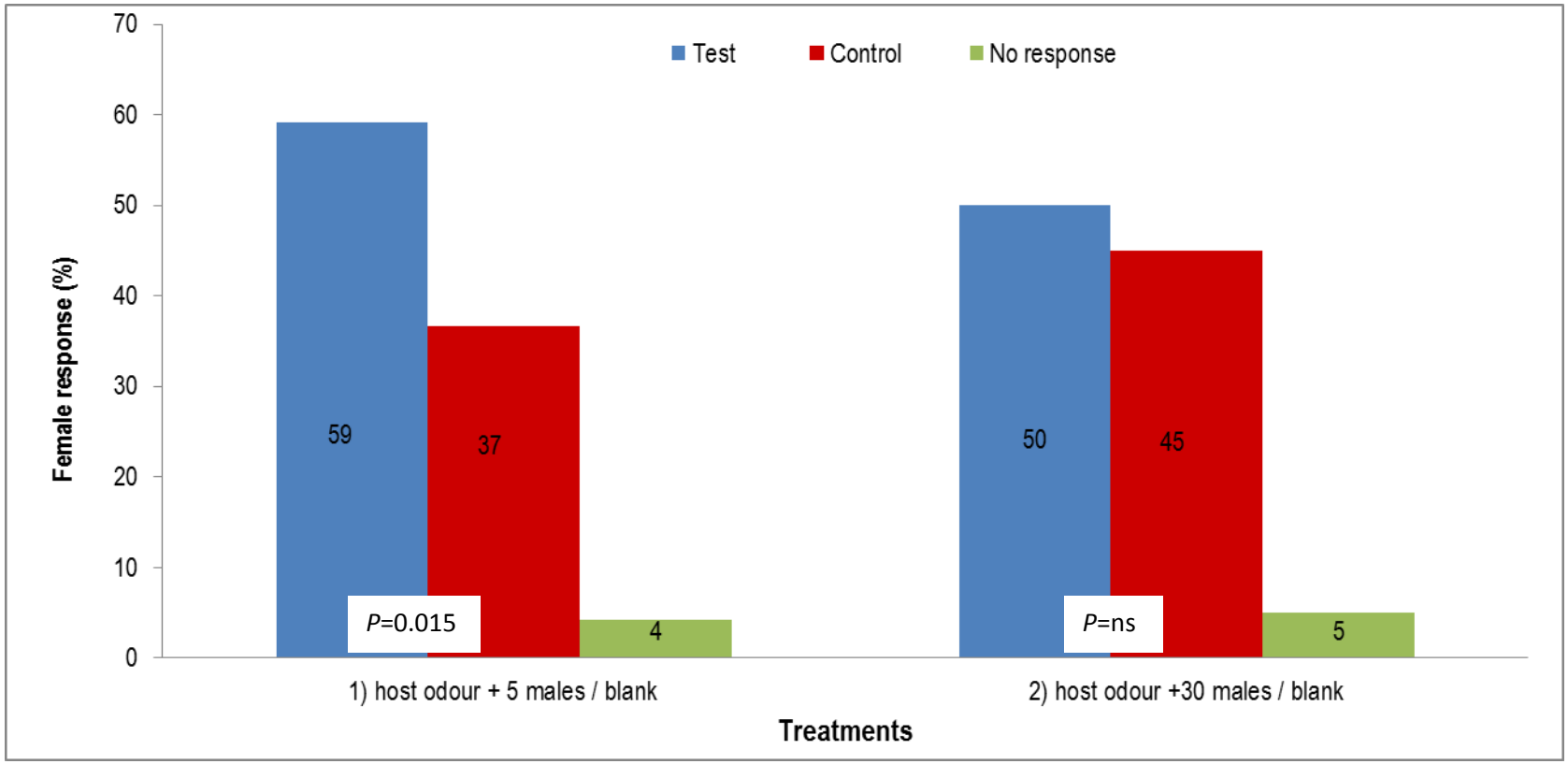

Figure 4.11: Percentage response of female *P. argentipes* (6d) (n=120) in a Y-tube olfactometer to: 1) host odour + males (n=5) (2d) / blank and; 2) host odour + males (n=30) (2d) / blank. *P*-value was calculated using a proportional exact test.

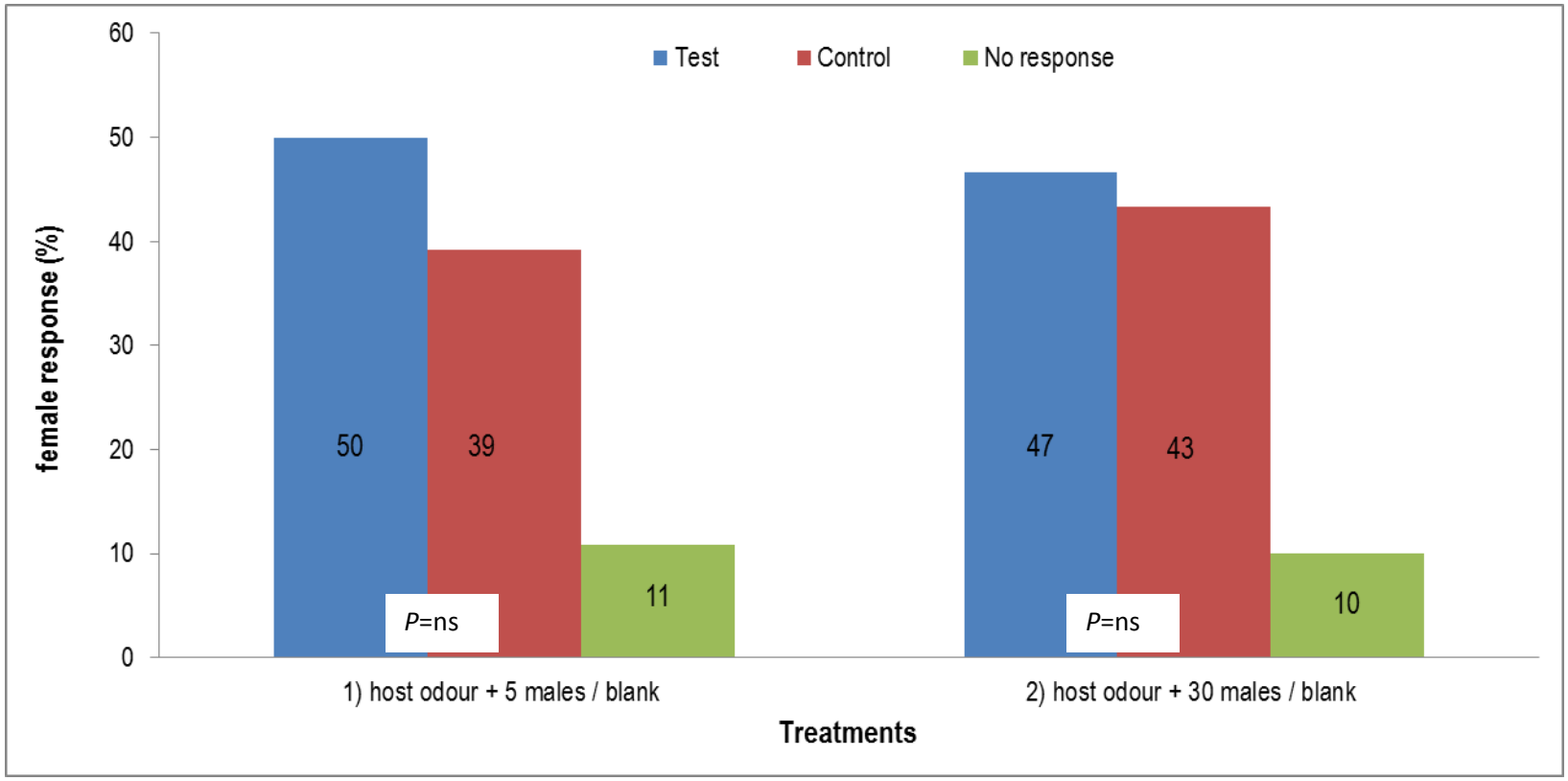

Figure 4.12: Percentage response of female *P. argentipes* (2d) (n=120) in a Y-tube olfactometer to: 1) host odour + males (n=5) (6d) / blank and; 2) host odour + males (n=30) (6d) / blank. *P*-value was calculated using a proportional exact test.

4.3.4 Response of Females (6d) to Hexane Extract of 30 Males (6d) *P. argentipes*

Females (6d) were significantly attracted to the hexane extract of 30 (6d) male *P. argentipes* in the presence of host odour (Figure 4.13) (1-Proportion exact test: number of females to test=59, number of females to control=37, *P*=0.032) but not when host odour was absent (exact test: test=50, control=50, *P*=ns).

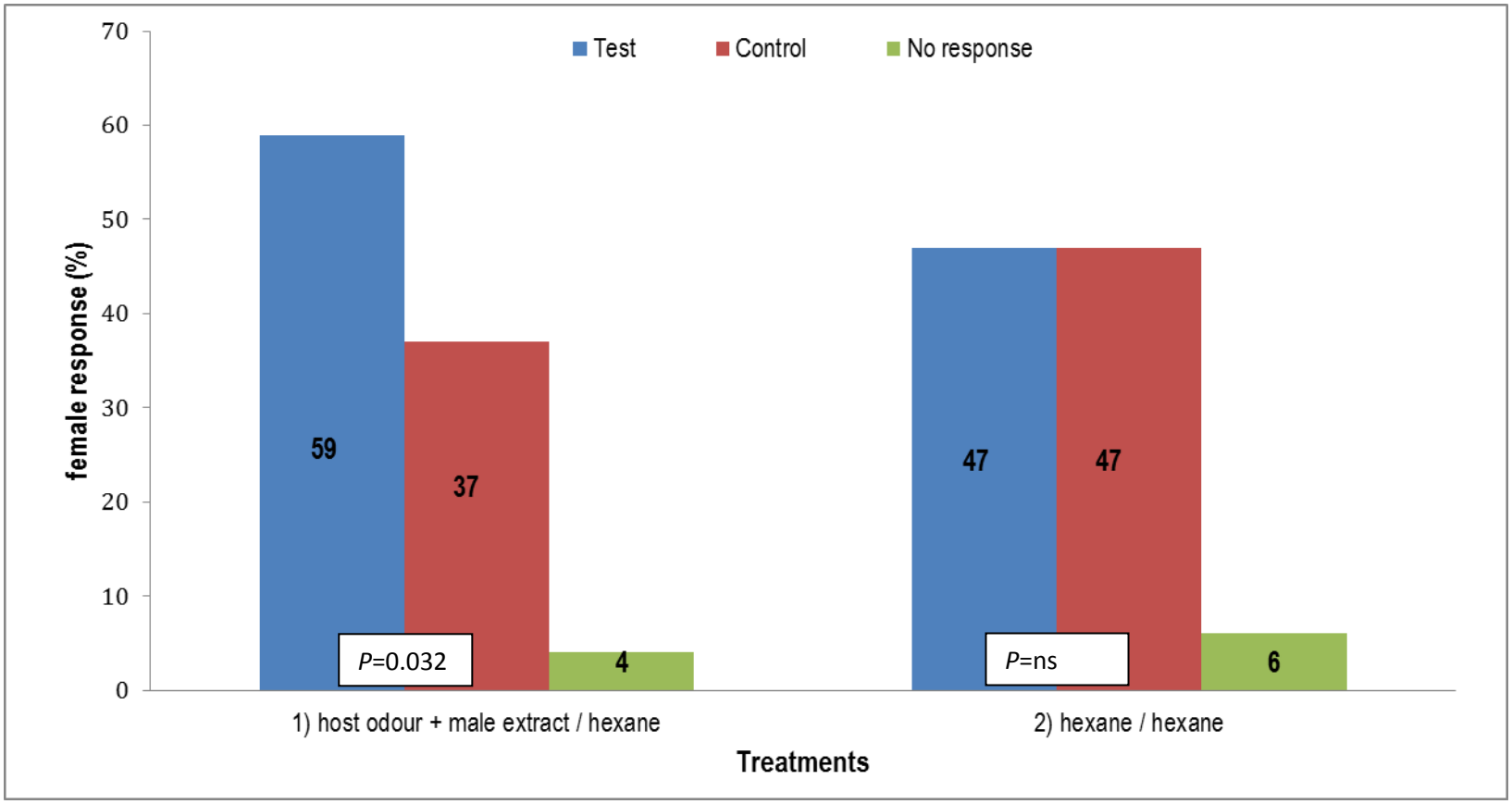

Figure 4.13: Percentage response of female *P. argentipes* (6d) (n=120) in a Y-tube olfactometer to; 1) host odour + male extract (6d)/hexane and; 2) hexane/hexane. . *P*-value was calculated using a proportional exact test.

4.3 DISCUSSION

The results presented in this chapter strongly suggest that a volatile male produced sex pheromone is present in *Phlebotomus argentipes*. Although this is not the first time that a sex pheromone has been suggested in an Old World species (Chelbi *et al.*, 2010) it is the first time that a behavioural response in a moving air assay has been observed for this important species. Other workers have suggested that *P. argentipes* males produce a sex pheromone (Kumar *et al*., 2012). However their results relied on the demonstration of contact behaviour to a filter paper disk rather than an upwind anemotaxis in an olfactometer that could be misinterpreted. In addition their results may have been skewed by the activity of a few highly active individual females and thus not be representative of the test population as a whole. The results presented here show that under certain circumstances of age of males and females and the presence of host odour that females are attracted to live male *P. argentipes* and organic solvent extract of male *P. argentipes*.

The preliminary experiments showed that age was an important factor in determining whether or not *P. argentipes* couples would mate. Older males (6d) and females (6d) mated more readily than younger males (2d) and females (2d). For this reason older males and females were used in the later experiments to determine response to large or small numbers of males and to extract of males.

In Y-tube olfactometer experiments, young (2d) male *P. argentipes* were significantly less attractive than older (6d) males. This observation is consistent with observations made in other species of sand flies e.g. *Lu. longipalpis* and *P. papatasi*. In *Lu. longipalpis* younger males produce less sex pheromone as they are observed to have fewer and smaller vacuoles in sex pheromone glands in the abdomen (Spiegel *et al*., 2002). The same may also be true for *P. papatasi* although no source of sex pheromone has yet been identified in that species.

Although newly emerged male and female sand flies are found ready to mate in the colony, results from these experiments revealed that there was no significant difference in the proportion of young female flies (2d) that responded in the Y-tube olfactometer to the arm containing the odour of young males (2d) or the blank arm in the presence of potential host odour. This finding suggests that the attractiveness of a sex pheromone produced by male *P. argentipes* could be dependent on the age of the responding female. Jones (1997) established that *Lu. longipalpis* females are attracted to and prefer to mate with middle-aged males (4-6 days old) rather than younger (12 hours – 2 days old) or older (8-10 days old) males. This is a choice that could be related to pheromone production (Jones *et. al.*, 2000). Jarvis and Rutledge (1992) also presented evidence to suggest that middle-aged (6-10 days old) males *Lu. longipalpis* were more successful in mating and females mated to older males laid more eggs than those mated to younger males.

A greater proportion of *P. argentipes* females were attracted to large

numbers of males (n=30) compared to small numbers of males (n=5) in the presence of host odour. Palit *et al*. (1993) and Lane *et al*. (1990) studying the aggregation behaviour of *P. argentipes* on animal hosts in India and Sri Lanka respectively, demonstrated that the proportion of males to females on the host animal ranged from between 8-27 males to one female. This suggests that a group of less than 8 males may be less attractive to females, and most probably a group of 30 males would be required to attract females to the vicinity of the potential host.

Young females (2d) were not attracted to older males (6d) in small or large groups. However old females (6d) were attracted to a small group of young males (2d) but were not attracted to a large group of young males. This result seems anomalous given the other results reported in this chapter and it might have been expected that a large group of young males would be attractive if a small group is attractive, and this should be investigated further. The lack of response of young females to old males suggests that young females may be sexually immature in their response to any sex pheromone released by older males. It has been suggested that mating activities are age-dependent (Boufana *et al*., 1986; Jarvis and Rutledge, 1992; Jones, 1997; Jones *et al*., 2000) and it may be that there is an interaction between age of females and size of the lek of males on or around the host that have been observed by many workers in the field (Lane *et al*., 1990; Palit *et al*., 1993).

A striking result of these experiments reported in this chapter was the

observation of the importance of the presence of host odour in inducing female *P. argentipes* to respond in the Y-tube olfactometer. There was a significant reduction in the numbers of females that did not respond in the bioassay experiment when host odour was present. As the potential hosts were not visible to the female sand flies from the Y-tube olfactometer and as the temperature was controlled equally for both arms, this suggested that host odour was important in stimulating or orientating female flies towards male sand flies. Those findings support the observed response of *Lu. longipalpis* virgin females to sex pheromone released by their conspecific males flies in the presence of the potential host (Bray and Hamilton, 2007). Those results and the results of others suggested that the host odour had a synergistic effect on the attractiveness of male sex pheromone. This finding indicates that presence of potential host is able to enhance the attractiveness of sex pheromone emitted by male flies to female flies for mating purposes before, while or after they blood feed on the host.

This chapter also showed that *P. argentipes* female flies were significantly attracted to hexane extract of males when compared to hexane alone in the presence of potential hosts. This signifies the possibility of replacing the presence of *P. argentipes* males around the hosts to attract females for mating and blood feeding purposes. This would be the first step taken to explore the possibility to develop a synthetic sex pheromone compound that equivalent to the sex pheromone produces by *P. argentipes* males. The usage of synthetic pheromone lures could possibly apply in an integrated vector control approach, additional to conventional control programme, in order to trap and kill female flies that transmit

leishmaniasis in a control and monitoring vector programme efficiently (Bray *et. al*., 2009).

CHAPTER 5: COURTSHIP BEHAVIOUR IN *PHLEBOTOMUS ARGENTIPES*

5.1 INTRODUCTION

Combining synthetic pheromone and insecticide, to attract and kill insects has been adopted in some vector control programmes (Oliveira-Filho and Melo, 1994; Kline, 2006, 2007; El Sayed *et al*., 2009; Bray *et al*., 2010). However it has been suggested that a comprehensive study of the courtship behaviour of these target insects, to better understand their mating behaviour, is needed so that the efficacy of such methods can be maximised (Kelly and Dye, 1997; Girling and Cardé, 2006; Bray and Hamilton, 2007).

Extensive studies of mating behaviour in sand flies has been undertaken, in particular to understand the relationship between the male sex pheromones and their effects on the behaviour of other males and females and mate choice between individuals and within populations, either in the laboratory or in nature (e.g. Kelly and Dye, 1997; Jones and Hamilton, 1998; Jones *et al*., 1998, 2000; Jones, 2001; Jones and Quinnell, 2002; Bray and Hamilton, 2007). Swarming, or lekking in sand flies, has been considered to be a strategy by which males attract females and to increase probability of mating success. Males are believed to

employ sex pheromones to achieve this. Acoustic signals may also be used during mating to communicate with intended mates over either short distance for recognition and stimulation e.g. *Drosophila* (Greenspan and Ferveur, 2000) or long distance for attraction e.g. cricket (Hedwig, 2006; Wagner and Basolo, 2007). In *Lutzomyia longipalpis*, an acoustic signal has been identified and it is now clear that at least three different types of copulation songs are produced by males during courtship depending on which of the populations in Brazil they are derived from (Ward *et al*., 1988; de Souza *et al*., 2002). There is evidence that sex pheromones of these acoustically different populations are also known to be different (Hamilton *et al*., 1996a, b).

Jarvis and Rutledge (1992) suggested that parading in male *Lu. longipalpis* was a display behaviour that enabled the male to mark his territory to either another male or female in a lek. Wing flapping by males may also disperse the sex pheromone to attract or arrest female or it may serve as a marker of male fitness in male-male competition and/or female choice (Ward *et al*., 1988; Lane *et al*., 1990; Jones and Hamilton, 1998). In *Phlebotomus papatasi*, it has been suggested that male wing flapping helps to move the aedegal filaments closer to the spermathecal ducts of female to be able to deposit the spermatophore (Ilango and Lane, 2000; Chelbi *et al*., 2012). A ‘courtship dance’ in *P. argentipes,* has been described by Palit *et al*. (1993) and it involves the male in short hops, swinging of his terminalia and wing flapping while on a host animal.

The series of individual behaviours that occur during courtship and the sequence of the behaviours, from the beginning until the end, between a pair of male and female sand flies have been studied in *Lu. longipalpis* and *P. papatasi* (Bray and Hamilton, 2007; Chelbi *et. al*., 2012). In these studies, details of the behaviour displayed were noted, and the sequences of the behaviours were analysed and positive and negative behaviours that could be seen during mating were also identified. These studies valuably illuminate the intricate interactions between males and females some of which may be critical for mating success. Understanding why some males or females are not successful whereas others are may offer opportunities for control, such as the suggestion that contact pheromones may be involved in male female interactions (Bray and Hamilton, 2007). Chelbi *et al*. (2012) found that in *P. papatasi*, female stationary wing-flapping and male copulation attempts are two behavioural indicators that indicate that courtship attempts will lead to successful mating. In *Lu. longipalpis*, Bray and Hamilton (2007) showed that male wing-flapping when nearly approaching female and a tendency to semi-circle females before attempting to copulate were two behaviours that successfully predicted mating success. Female behaviour is also important and female *P. papatasi* tend to rub her legs when she is not receptive to the males, this behaviour was not observed in *Lu. longipalpis*.

Observations of *P. argentipes* mating behaviour that have been described by Palit *et al*. (1993) were mainly on the behaviour of males in a lek and during copulation on the host. These are more qualitative description of individuals and pairs and so lack any relationship to which are important in the population as a

whole. Thus, quantitatively, there is still lack of the exact individual behaviour that may be displayed by both males and females during courtship. As there is evidence of chemical signals involved in the mating behaviour of *P. argentipes*, i.e. females are attracted to male-produced sex pheromone over a distance in a Y-tube olfactometer bioassay (Chapter 4), it was hypothesised that there could be an association between behaviour displayed and information transferred between them during courtship. Knowledge of this could be used to strengthen our understanding of the basic biology of *P. argentipes* and may direct us towards future research avenues leading to new vector control tools.

This study is aimed to provide an accurate, detailed and quantitative description of courtship in *P. argentipes,* in which the interactions of pairs of sand flies were observed under laboratory conditions. The differences in the behaviour displayed by both the male and the female were described and analysed to quantify the behaviour. The interactions between them were analysed to determine the sequence of behavioural events and specifically which behaviours were significant because they led to subsequent behaviours, which led to copulation. The behaviours that were recognised and quantified were based on observations and descriptions in previous studies of *Lu. longipalpis* (Ward *et al*., 1988; Jones and Hamilton, 1998; Bray and Hamilton, 2007), *P. papatasi* (Chelbi *et al.,* 2012), *P. argentipes* (Lane *et. al*., 1990; Palit *et al*., 1993) and other insects (Kamhawi *et al*., 1992; Gebre-Michael *et al*., 1994; Mahamat and Hassanali, 1998; Barbour *et al*., 2007; Casares, 2007). Analyses of the behaviour were to determine

the behaviour(s) displayed by either male or female that are vital to mating success.

5.2 MATERIALS & METHODS

Adult males and females were from the colony maintained as previously described in Chapter 2. Male and female 6d flies used in the observations were separated within 5 hours of emergence before the rotation of the external male genitalia. They were fed only on saturated sugar solution in an adult holding cage. Two separate cages consisting of ten flies of each sex were acclimatised to experimental condition for an hour prior to trials. All of the observations were carried out on the solid, vibration-damped bench in the bioassay room at 27°C±2°C and 85% relative humidity under white fluorescent light between 1400 and 1800 hours.

5.2.1 Recording of the Courtship Behaviour

The courtship interactions between 40 individual pairs of *P. argentipes* males and females were observed in a courtship arena consisting of a round plastic container (diameter 22 mm and height 15 mm) with a 10 mm layer of plaster of Paris at the bottom and surrounding wall surface. The top of the arena was covered with a glass slide (76 x 26 x 1 mm) to prevent flies from escaping and to enable recording of the interactions between the couples. The size of arena was chosen to facilitate movement of male and female sand flies during courtship and also to enable the interactions to be recorded sufficiently. All observations were carried out in a temperature-controlled bioassay room and constantly maintained

at 27°C±2°C under a white fluorescent light. A fibre optic light source (KL 500; Schott UK Ltd, Stafford, UK) was used for additional illumination during filming.

Behavioural interactions were recorded using a colour video camera (TK-1280E; JVC, London, U.K.) fitted with a zoom lens (Computer 18-108 mm, f 2.5 manual focus; CBC (Europe) Ltd, London, UK) and supported 30 cm above the courtship arena using a copy stand (CS-920; Tracksys Ltd, Nottingham, U.K.). Output from the camera was fed through a vertical interval time code (VITC) generator (AEC-BOX-18; Adrienne Electronic Corp., Las Vegas, NV, USA) to a time-lapse security video recorder (VCR) (HS1024; Mitsubishi Electric, Hatfield, U.K.) set to non-stop recording. A feed from the VCR was sent to a colour monitor (Trinitron KV-14MIU; Sony, Thatcham, U.K.) to enable camera adjustments and to watch observations while filming.

For each observation, a male fly was removed from the cage using a mouth aspirator and placed into the arena via a notch cut into the glass slide. After a period of 5 min, the VCR was set to record and a female was placed into the arena using the aspirator. Males were placed in the arena earlier than the females to replicate natural sand fly behaviour i.e. males are reported to arrive earlier and establish their territories on or near the host before the females arrive (Lane *et al.*, 1990; Dye *et al.*, 1991; Jarvis and Rutledge, 1992). Observations of one male and female at a time were made until copulation occurred or for a maximum of 15 min where copulation did not occur. The courtship arena was replaced between

replicate. After use the courtship arena was washed with hexane and the glass slides were cleaned with 5% Teepol L detergent (VWR International, United Kingdom), distilled water and acetone.

5.2.2 Courtship Behaviour Analysis

Courtships behaviours recorded during observations were analysed using a PC fitted with a PC-VITC card (Adrienne Electronic Corp.) running the Observer Base Package for DOS (Version 3.0) and Support Package for Video Tape Analysis (Version 3.1; Noldus Information Technology, Wageningen, the Netherlands). Videotapes were played back using the same VCR that was used to record observations and the output was sent simultaneously to the PC-VITC card and the Sony TV monitor. Behaviour of both male and female sand flies was coded into mutually exclusive categories (in which only one of the behaviours listed in Table 5.2 could be performed by each fly at any given time) and entered into the Observer software via a sequence of key presses during video playback. Video images were replayed in slow motion and analysed with key presses in Observer synchronised to the time code recorded onto the video by the VITC generator, as read by the PC-VITC card.

Sequences of each pair of male and female courtship behaviours obtained from the Observer software were used to calculate the frequency and duration of the behaviour performed during observations and formed the basis of subsequent analysis of behavioural transitions. The differences in the frequency or duration of

behaviours performed by males and females were compared and statistically tested using the paired t-test or Wilcoxon signed rank tests, as appropriate. Fisher's exact test was used to establish which behaviour of male and female that lead to copulation, occurred most frequently compared to unsuccessful courtship interactions. Any behaviour displayed by either male or female that showed significant results was identified as a precursor to mating success.

5.3 RESULTS

5.3.1 Courtship Behaviour

40 pairs of *P. argentipes* males and females were observed individually during courtship. A series of individual behaviours performed by males and females were observed and categorised as in Table 5.1. Not courting (behaviour 1; Figure 5.1), where the sand fly remained stationary without any activity; was usually used as separator from one behaviour to other behaviours. Overall time spent on courtship activities (excluding time spent for copulating); males were found to be significantly more active during courtship than females i.e. mean time for male (s): 102.8±22.0, female: 46.8±11.4; (paired t-test) t_{39}=2.561, *P*=0.01).

Stationary wing flapping (behaviour 2; Figure 5.2) was observed to be performed by both male and female sand flies. Males significantly spent more time in stationary wing-flapping which lasted longer (6.39s) than females (2.00 s) (mean time for male (s): 5.7±0.69, female: 1.8±0.20; (Wilcoxon Signed rank test) Z=11.535, *P*<0.01). Males were also found to significantly more frequently stationary wing-flap compared to females (male: 14.4±2.38, female: 7.5±1.87; (Wilcoxon Signed rank test) Z=3.162, *P*=0.002).

Both male and female sand flies touched each other with the tips of their legs or antennae. The duration of time spent touching (behaviour 3; Figure 5.3a and Figure 5.3b) was not significantly different between males and females (mean

time for male (s): 2.3±0.41, female: 1.6±0.29; (Wilcoxon Signed rank test) Z=2.513, *P*=0.12). There was also no significant difference in the frequency of touching behaviour between male and female (male: 4.3 ± 0.73; female: 2.6 ± 0.46; (Wilcoxon Signed rank test) Z=1.803, *P*=0.071).

Another courtship behaviour performed by both male and female was dipping (behaviour 5; Figure 5.5a and Figure 5.5b) in which male or female moved their abdomen vertically pointing it to the surface of the courtship arena, a movement that happened very quickly. Although the female was found to spend significantly more time dipping than the male (mean time for male (s): 2.0±0.41, female: 4.3±0.50; (Wilcoxon Signed rank test) Z=4.015, *P*<0.01), there was no significant difference in the frequency for either of them (male: 0.9±0.29; female: 1.8±0.63; (Wilcoxon Signed rank test) Z=1.001, *P*=0.317).

Circling and dipping (behaviour 6; Figure 5.6) was observed in both *P. argentipes* males and females. In this behaviour the sand fly plunged its head and thorax to the surface of the arena, followed by dipping its abdomen in a circular or semi-circular movement around the same spot. This was often seen in a clockwise followed by an anti-clockwise direction and looked like a ‘spinning crazily’ movement, with the individual circling and dipping on the floor of the arena when observed from the top. There were no significant difference in the time spent or the frequency of the behaviour in males or females (mean time for male (s): 7.9±1.53, mean time for female: 12.6±2.07; (Wilcoxon Signed rank test) Z=1.903, *P*=0.057),

(frequency: male: 0.3±0.14; female: 0.4±0.14; (Wilcoxon Signed rank test) Z=1.185, *P*=0.236).

Facing (behaviour 4; Figure 5.4) was performed by the male and female concurrently. In this behaviour males and females faced each other and remained unmoving for a few seconds. Time spent facing per trial varied from 2.72 s to 4.28 s and mean frequency of facing for each trial was 0.7±0.16.

Males exclusively displayed three behaviours including approach-flapping (behaviour 8; Figure 5.8), abdomen bending (behaviour 9; Figure 5.9), and copulation attempt (behaviour 10, Figure 5.10). The duration of time spent abdomen bending per trial was from 2.17 s to 5.43 s and frequency of abdomen bending per trial was 0.93±0.29. Time spent approach-flapping per trial was from 1.93 s to 3.47 s and frequency per trial was 2.0±0.44. Duration of time spent in copulation attempt per trial was from 0.69 s to 1.91 s and number of frequency per trial was 1.3±0.61.

In total, 17 pairs (42.5%) out of 40 copulated successfully and 23 pairs (57.5%) did not. Only one male (5.9%) successfully copulated at the first attempt but 8 males (47.9%) copulated successfully at the second attempt and 5 males (29.5%) succeeded at the third attempt. The remaining males succeeded at their fourth, fifth and sixth attempts respectively. The duration of copulation i.e. the time

taken from when the male and female started to copulate until either the male or female pulled away, ranged from 7.3 min to 11.7 min.

Table 5.1: Behaviours observed during *P. argentipes* courtships.

No.	Name of behaviour	Description
	Male and female behaviours	
1	Not courting (*Fig. 5.1*)	Sand fly remains stationary without moving, wing-flapping, facing or touching its partner.
2	Stationary wing-flapping (*Fig. 5.2*)	Sand fly remains stationary and flaps both wings simultaneously. Flapping followed a pattern of small vibrations through a slight rotation of the wings followed by a large flap, in which both wings extended to an angle of 45–70^{o} from the body.
3	Touching (*Fig. 5.3a,b*)	Sand fly makes contact with its partner usually by touching with the tips of the legs or antennae, and sometimes on the surface of its abdomen.
4	Facing (*Fig. 5.4*)	Male and female face each other in close proximity for a few seconds.
5	Dipping (*Fig. 5.5a,b*)	Sand fly moves vertically by dipping its abdomen to touch the floor of the arena, often in a repeating pattern.
6	Circling and dipping (*Fig.5.6*)	Sand fly positions its head towards the arena floor and dips the end of its abdomen while moving in a circle or semi-circle around the same spot. Movement occurred in both clockwise and anticlockwise directions.
7	Copulation (*Fig. 5.7*)	Male and female copulate with the tips of the abdomen joined and facing in opposite directions. Males often flapped their wings until female appeared to accept copulation. Females normally remained motionless but occasionally struggled during copulation.
	Male-only behaviours	
8	Approach-flapping (*Fig. 5.8*)	Male vigorously flaps his wings and steps towards female in an alternating repeating pattern.
9	Abdomen bending (*Fig. 5.9*)	Male bends his abdomen laterally; swinging his terminalia to the left and right, commonly when female is nearby.
10	Copulation attempt (*Fig. 5.10*)	From a position parallel to the female, male bends his abdomen in an attempt to make contact with the female genitalia, often while wing-flapping.

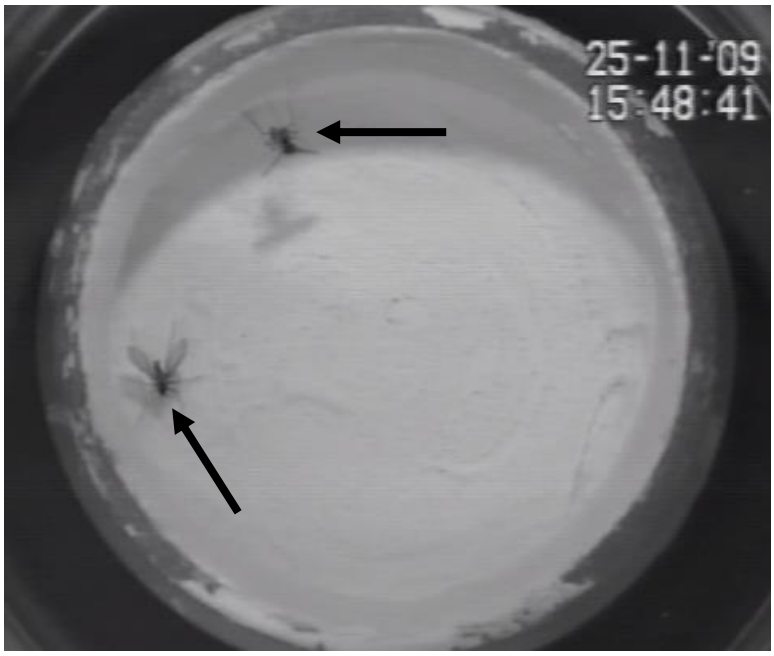

Figure 5.1: Not courting; both sand flies (indicated by the arrows) remain stationary without moving, wing-flapping, facing or touching their partner.

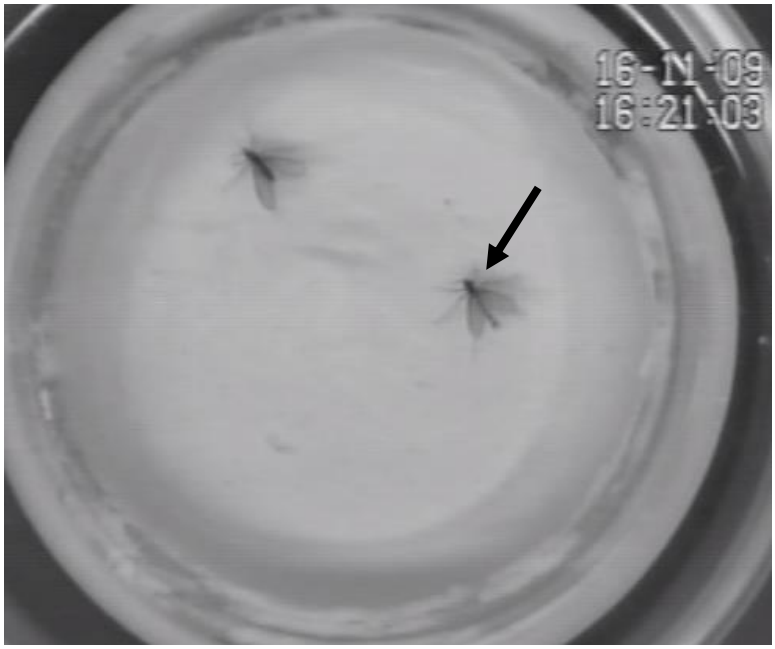

Figure 5.2: Wing flapping; male sand fly (indicated by the arrow) is stationary wing flapping.

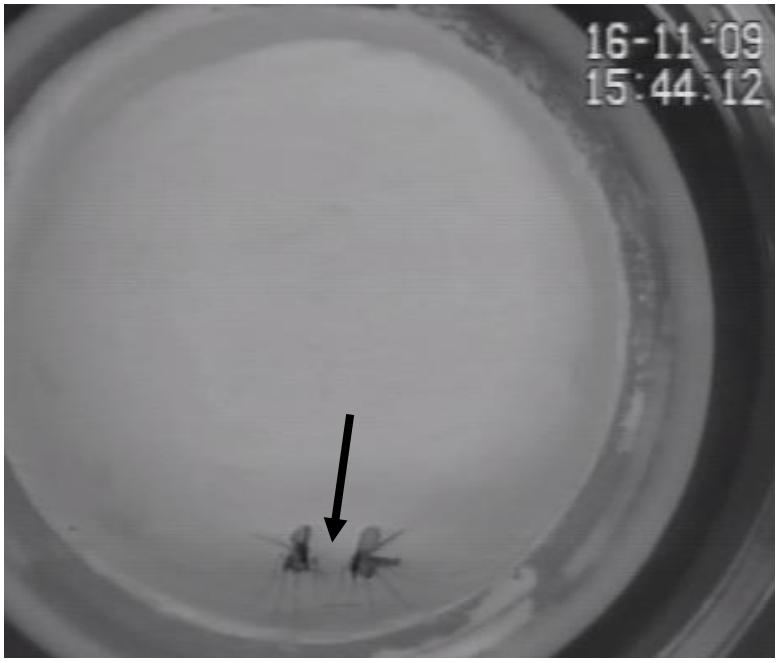

Figure 5.3a: Touching; sand fly makes contact by touching its partner with the tips of its legs.

Figure 5.3b: Touching; sand fly makes contact by touching its partner with the tips of its antennae.

Figure 5.4: Facing; male and female sand fly facing each other closely for a few seconds.

Figure 5.5 a: Dipping; female sand fly (on the right) moves vertically by dipping her abdomen to the floor of the arena.

Figure 5.5 b: Dipping; male sand fly (on the right) moves vertically by dipping his abdomen to the floor of the arena.

Figure 5.6: Circling and Dipping; female is circling and dipping.

Figure 5.7: Copulation;male and female copulate when the tips of their abdomen joined.

Figure 5.8: Approach flapping; the male wing flaps and steps towards the female in an alternating repeating pattern.

Figure 5.9: Abdomen bending; male bends its abdomen laterally towards female.

Figure 5.10: Copulation attempt; male bends its abdomen attempting to copulate with female.

5.3.2 Courtship Behaviour as a Predictor of Copulation Success

In the courtship trials, certain male behaviours played an important role in determining the success of copulation attempts and thus could be considered as predictors of success. Specifically those behaviours were the male flapping his wings vigorously while walking toward female, and then, while in the vicinity of the female, the horizontal swaying of his genitalia followed by a copulation attempt whilst parallel to the female. These behaviours were performed significantly more frequently by males who successfully copulated when compared to unsuccessful males (Table 5.2). Males (82.5%) that had successful courtships performed approach wing-flapping behaviour (Figure 5.8) in contrast to 34.8% whose courtships were unsuccessful. Also 52.9% of males performed abdomen bending (Figure 5.9) in successful courtship compared to 17.4% involved in unsuccessful courtships. The most important behaviour that predicted successful courtship was 'copulation attempt' (Figure 5.10), 94.1% of males that attempted copulation were successful compared to only 4.3% of males that did not (Table 5.2).

P. argentipes male also displayed a behaviour that very reliably predicted an unsuccessful courtship. Dipping behaviour (Figure 5.5a and Figure 5.5b), in which the male moved his abdomen vertically onto the surface of the arena, was carried out by 56.5% of males that failed to copulate with their partners, in contrast only 5.9% of males that displayed dipping behaviour successfully copulated with their partners (Table 5.2). Although females also displayed the same behaviour, there was no significant difference in the number that displayed it in successful

compared to unsuccessful courtships. Circling and dipping behaviour (Figure 5.6) occurred in 26.1% of males that were unsuccessful in courtship and did not occur at all in successful males (Table 5.2). Again, although females also performed this behaviour it did not predict female courtship success.

Stationary wing-flapping (Figure 5.2), touching (Figure 5.3a and Figure 5.3b) and facing (Figure 5.4) behaviours were commonly displayed by both males and females. These behaviours showed statistically no different in the frequency of occurrence in either successful or unsuccessful courtships (Table 5.2).

Table 5.2: The numbers of courtship behaviours occur in successful and unsuccessful courtships for each individual.

	Courtship Behaviours	Successful courtship [N=17] (%)	Unsuccessful courtship [N=23] (%)	*P* value
	Male behaviours:			
	Stationary wing-flapping	17 (100)	22 (95.7)	1.00
	Touching	15 (88.2)	21 (91.3)	1.00
	Facing	6 (35.3)	10 (43.5)	0.747
§	Dipping	1 (5.9)	13 (56.5)	**0.012***
§	Circling and dipping	0	6 (26.1)	**0.030***
†	Approach wing-flapping	14 (82.4)	8 (34.8)	**0.004****
†	Abdomen bending	9 (52.9)	4 (17.4)	**0.038***
†	Copulation attempt	16 (94.1)	1 (4.3)	**< 0.005****
	Female behaviours:			
	Stationary wing-flapping	11 (64.7)	14 (60.9)	1.00
	Touching	10 (58.8)	19 (82.6)	0.153
	Facing	6 (35.3)	10 (43.5)	0.747
	Dipping	6 (35.3)	13 (56.5)	0.216
	Circling and Dipping	2 (11.8)	6 (26.1)	0.428

†Behaviour more likely to lead to successful courtships. (Fisher's exact test)
§ Behaviour more likely to lead to unsuccessful courtships.
Significant difference at: *$P<0.05$, **$P<0.01$

5.4 DISCUSSION

The courtship behaviour of *Phlebotomus argentipes* comprises of a series of individual behaviours of male and female sand flies both of which actively participate. The core progression of behaviours comprised the male wing-flapping while approaching the female, before touching her with the legs or antennae prior to attempting copulation. This builds on a previous description of the 'courtship dance' of *P. argentipes*, described as involving males hopping, swinging the terminalia and wing-flapping (Palit *et al.*, 1993).

Some of the behaviours displayed by *P. argentipes* are similar to those described in other Old World (*P. papatasi*) and New World (*Lutzomyia longipalpis*) species. In both *P. argentipes* and *P. papatasi* (Chelbi *et al*., 2012) stationary wing-flapping, touching and facing are performed by males and females. However in *Lu. longipalpis* only stationary wing-flapping and touching behaviour are seen (Bray and Hamilton, 2007).

Wing-flapping is commonly seen in the mating behaviour of sand flies (Ashford, 1974; Ward *et al*., 1988; Bray *et. al*., 2010). However, even though stationary wing-flapping was frequently observed among *P. argentipes* males and females, it is not a predictor of successful mating for either sex. This is unlike the situation in female *Lu. longipalpis* and *P. papatasi* where, interestingly, stationary wing-flapping during courtship indicated that the courtship would lead to

successful mating. In male *Lu. longipalpis*, although wing-flapping is not an indicator of successful mating, it is believed to be an act to disseminate the sex pheromone. It is possible that the act of wing-flapping in *P. argentipes* males has a role that is comparable to a suspected role of wing-flapping in *Lu. longipalpis* i.e. to disseminate the sex pheromone. Further investigations are needed to reveal and confirm this matter for *P. argentipes*.

In addition to chemical communication, *P. argentipes* wing-flapping may also function in production of audio signals important to mating. Courtship songs, produced by rhythmic wing vibrations are believed to play a role in species recognition in *Lu. longipalpis*, as the pattern of sound produced by males during copulation differs between members of the species complex (Souza *et al*., 2004, 2008). Similar audio signals have also been recorded during courtship in *Lu. intermedia* (Vigoder *et al*., 2011), and during copulation in *Lu. cruzi* (Vigoder *et al*., 2010a) and *Lu. migonei* (Vigoder *et al*., 2010b). Audio signals have recently been recorded from *P. argentipes* (Hamilton and Brazil, 2015 unpublished) and it is therefore likely that they are widespread in other Old World species where wing-flapping has been noted (Bray *et al*., 2010; Chelbi *et al*., 2012). As in *P. papatasi*, male *P. argentipes* flapped their wings only briefly at the start of copulation (Chelbi *et al*., 2012), possibly to assist in alignment of the male and female genitalia. This may suggest that audio signals produced prior to copulation (rather than during) may play a greater role in courtship. Manipulative playback experiments, similar to those carried out in Drosophila (Talyn and Dowse, 2004) are needed to determine the function of audio signals (if any) in sand fly mating behaviour.

Both male and female *P. argentipes* were observed to physically contact each other by touching their partners, most commonly on the legs or the antennae. This behaviour has also been reported from studies of *P. papatasi* and *Lu. longipalpis* (Bray and Hamilton, 2007; Chelbi *et al*., 2012). Whilst found to be an integral part of the behavioural progression towards copulation, occurrence of this behaviour does not in itself predict copulation success in any of the three species examined to date by Bray and Hamilton (2007); Chelbi *et al*. (2012) and also Bray *et al*. (2014).

Although the function of this touching behaviour is unknown in sand flies, it has been explained in detail in other insects. In *Drosophila melanogaster*, a layer of chemicals on the cuticle, known as cuticular hydrocarbons, were shown to be involved in mating via physical contact (Casares, 2007) and in the long-horned beetle, cuticular hydrocarbons mediate mate recognition (Barbour *et al*., 2007). In sand flies, including *P. argentipes*, differences in cuticular hydrocarbons can be used to distinguish between populations and species (Kamhawi *et al.,* 1992; Gebre-Michael *et al*., 1994; Mahamat and Hassanali, 1998). There is lack of evidence that shows exchange of cuticular hydrocarbons between male and female *P. argentipes* however a recent study examined in detail the cuticular hydrocarbon profiles of male and female *P. argentipes* and there were significant differences between the sexes. Comparison of retention times with straight chain alkanes suggested that the female-associated chemicals may be smaller than the C20–C40 chemicals normally recovered from cuticle wax (Phillips and Milligan,

1986), whereas the male-associated compounds appeared to be in the normal range for cuticular hydrocarbons. Differences in the chemical profiles of males and females, and a potential behavioural mechanism for transmission and reception of these chemicals (touching) suggest the presence of a sex pheromone. However, this is not in itself evidence for sex pheromones and more work is required to identify the potential chemicals involved, and to conduct bioassays to ascertain their relevance to mating and other behaviour. In particular, experiments are needed to determine whether the male-associated chemicals detected here could be responsible for the response of female *P. argentipes* to male extracts observed in Chapter 4.

During courtship, male and female *P. argentipes* were observed to move their abdomen vertically, dipping it onto the surface of the courtship arena. Analysis revealed that male *P. argentipes* could use this as a signal of unwillingness to mate. When this occurred, copulation was significantly less likely to occur. Similar abdomen dipping behaviour has previously been observed in female *Lu. longipalpis*, which are free to choose from a number of potential mates within a lek (Jones and Hamilton, 1998). However in males this behaviour is unique to *P. argentipes* it has not been reported either in *P. papatasi* or *Lu. longipalpis*. It has been suggested that in *Lu. longipalpis* females that this behaviour is linked to monandry as for the female the correct mate choice is essential. However, why male *P. argentipes* should reject a potential mate is unclear, as males make relatively little contribution to offspring production. As only virgin males were used in this study, sperm depletion is also unlikely to explain this

result. Another behaviour unique to male *P. argentipes* courtship is circling and dipping. The sand fly first, plunges his head to the floor and then follows that by dipping the end of his abdomen while moving in circular or semi-circular around the same spot, usually in a clockwise and anti-clockwise direction. This behaviour, when seen from above, looks like the sand fly is spinning its whole body in a clockwise and then anti-clockwise direction interchangeably, and very rapidly. Whenever the male displayed this behaviour it indicated that he was not interested in mating with his partner. Further work is needed to ascertain whether rejection of females is a genuine feature of mating behaviour of *P. argentipes*, or an artefact of the trial conditions. If chemically mediated, mate rejection could form a target for mating disruption as a means of vector control.

Other *P. argentipes* male only behaviours that include; approach wing-flapping, abdomen bending and attempt to copulate, were very much alike those previously observed in *P. papatasi* (Chelbi *et al*., 2012) Abdomen bending is not seen in *Lu. longipalpis*, but the other two behaviours, approach wing-flapping and attempt to copulate are seen (Bray and Hamilton, 2007).

The courtship behaviours displayed by *P. argentipes* males resembled those that had been observed by Palit *et al*. (1993) on a hamster in a laboratory experiment. In that study they described that when a *P. argentipes* male closely approached a *P. argentipes* female, he bent his terminalia horizontally to the left

and right of his body, while flapping his wings vigorously in a manner similar to that described in *P. orientalis* (Ashford, 1974) and *Lu. longipalpis* (Ward *et al*., 1988).

They assumed that males were displaying a ‘courtship dance’ that included short hops, wing-flapping (steps towards the female while flapping his wings) and abdomen bending, before copulating happened. They found that in all of the observations of successful mating, the ‘courtship dance’ of males occurred, but no ‘courtship dance’ or any courtship behaviours by females were reported.

In this study, once copulation started, the *P. argentipes* male commonly remained motionless as did the female this behaviour lasted for from 14 s to 32 min. It ended when either the male or female pulled away from copulation. Sometimes the male wing-flapped until the copulation was stable i.e. when the female remained motionless and finally accepted copulation. Sometimes the male wing-flapped while moving around the arena with the female still in copula. However, Palit *et al*. (1993) recorded that copulation had occurred in the range from 4 s to 3 min 47 s, which was less than in this study. This difference may have been due to the presence of the host kairomone that synergised the male pheromone making the male more attractive to the female (Palit *et al*., 1993). A further difference is that in those experiments *P. argentipes* females could choose their potential male freely among the other lekking males. In this study the host was not present and female was not given a choice of choosing its potential mate in essence the male was chosen randomly and placed in the mating arena. It

would be very interesting to carry out a further series of experiments in a bigger arena and in the presence of host odour to determine how these mating interactions might differ under the presence of different numbers of males and females where the males were forced to compete with each other for mating and also females were forced to compete for the available males. However the experiments reported here clearly indicate which behaviours are critical to mating success.

Interestingly, in *P. argentipes,* female courtship behaviours were not significant in leading to successful mating this is unlike the situation in *P. papatasi* and *Lu. longipalpis* where stationary wing-flapping leads to successful courtship. Only *P. argentipes* male behaviours during courtships were considered crucial to a successful mating. Whilst dipping and also circling and dipping were behaviours to indicate failure to mate, approach wing-flapping, abdomen bending and copulation attempt were usually associated with mating success. Generally, in insect mating systems, males are considered to be more prepared to court and copulate than females because time and sperm donated are their main mating cost. In contrast females are commonly found to be very selective in their mate choices (Thornhill and Alcock, 2001) this is likely to be due to their investment in time and resources to producing the next batch of offspring.

P. argentipes mating behaviour is very similar to *Lu. longipalpis* mating behaviour in that males of both species appear to lek on or near the animal host

(Ward, 1988; Lane *et al*., 1990; Palit *et al*., 1993). They also reported that the male wing flapping, which is usually performed in short pulses, is similar to that seen in *Lu. longipalpis* and we now know that this wing fluttering produces a “courtship song” that is similar but different to the songs produced in the *Lu. longipalpis* species complex (Hamilton and Brazil, 2015 unpublished). This courtship song is likely to be a very important part of the initiation and maintenance of successful mating in both *P. argentipes* and *Lu. longipalpis*.

P. argentipes males were observed to be likely to bend their abdomen to nearby females before their attempt to copulate, this behaviour has not been observed in *Lu. longipalpis*, however it was in *P. papatasi*. All three species of sand flies; *P. argentipes*, *P. papatasi* and *Lu. longipalpis*, share one common behaviour, copulation attempt and this is critical to mating success in all three species.

Very little is known about the mating strategy of *P. argentipes* and this is the first quantitative study of courtship behaviour in this important vector species. Experiments to answer questions such as whether females mate only once or more often, or why males appear to reject females are essential for developing and understanding of a key life history stage of this insect and for the development of new and innovative control strategies. The results of this study demonstrate that courtship in *P. argentipes* shares similarities with both the new world VL vector *Lu. longipalpis*, and the Old World cutaneous leishmaniasis vector *P. papatasi*. As

wing flapping seems crucial to mating success in this species, future studies should attempt to identify the modality of the signal produced by this behaviour and in particular its role in the courtship behaviour for example whether or not it is a pre- or post-zygotic mating barrier. In *Lu. longipalpis* the range of courtship songs produced by males appear to be indicative of different members of the species complex; it would also be interesting to determine and if the *P. argentipes* courtship song varied in a similar way across the full range of *P. argentipes* distribution. There may also be opportunities for exploitation of the courtship song as a means of vector control which should be explored in due course. Touching is an important behaviour displayed by both males and females and may be associated with detecting specific chemicals on the cuticle of the partner.

Chemical analyses and behavioural bioassays are now required to identify the chemicals present on the surface of male and female *P. argentipes*, and to determine if they have any role in attracting or dissuading potential mates. Both sexes of *P. argentipes* reject potential mates, which suggests that some individuals are more attractive than others. *Lu. longipalpis* females are known to prefer a small number of males within an aggregation, and attractiveness in this species is both an inheritable characteristic, and associated with pheromone production (Jones and Hamilton, 1998; Jones *et al*., 1998). Identifying differences between relatively attractive and unattractive individuals in *P. argentipes* would be a logical next step in identifying the modality of sexual signals used in this species.

The study in this chapter revealed a series of interactions between *P.*

argentipes males and females that involved cues and responses between them that lead to mating success. The full range of cues could involve chemical, tactile, vision or acoustic signals or encompassing two or more signals, and these should be investigated further.

CHAPTER 6: GENERAL DISCUSSION

6.1 PRINCIPAL FINDINGS

The overall aim of this study was to determine if volatile chemicals involved in oviposition and mating might be present in *Phlebotomus argentipes* and by a detailed examination of the courtship behaviour of both sexes determine if non-volatile chemical cues might also be important. Specific objectives were to; 1) determine if an oviposition pheromone was associated with eggs; 2) determine if the oviposition pheromone was present on the surface of eggs; 3) establish in an moving air olfactometer if a volatile sex pheromone is produced by male *P. argentipes*; 4) determine the age of males that produce this cue and the age of females that respond to it; 5) determine if the sex pheromone can be removed from male *P. argentipes* in an organic solvent and used to induce a response by females in a Y-tube olfactometer and; 7) establish a detailed description of courtship behaviour of *P. argentipes* to determine if other non-volatile chemical cues and behaviours might be critical to successful mating.

6.1.1 Oviposition Response to Oviposition Pheromone Associated with Conspecific Eggs

The presence of an oviposition pheromone associated with conspecific eggs was investigated using behavioural bioassays. It was found that gravid *P. argentipes* laid their eggs in the vicinity of a site where conspecific eggs had previously been placed. This type of response has been previously observed in other species of sand flies e.g. *Lutzomyia longipalpis* (El Naiem and Ward, 1991; El Naiem *et al*., 1991; Dougherty *et al*., 1992, 1994; Dougherty and Hamilton, 1997) and *P. papatasi* (Srinivasan *et al*., 1995), where gravid females are stimulated to lay more eggs in the vicinity of previously deposited conspecific eggs. There is no evidence presented here or by the previous workers to suggest that the eggs are attractive to gravid females and it would be very interesting to conduct a series of olfactometer experiments to investigate this issue.

It was also noted that gravid *P. argentipes* females seem to lay their eggs in alternative sites when the number of eggs present on the initial site reaches a threshold. This suggests that there may be a maximum number of eggs which can be laid at any one site and this information could be relayed by high concentrations of pheromone (i.e. when greater number of eggs are present), stimulating females to find other sites to lay their eggs (El Naiem and Ward, 1991; Dougherty *et al*., 1994). It is suggested that this behaviour may be a mechanism to avoid over populating a habitat.

The observation that gravid females response to previously laid whole eggs with further egg laying suggested that a pheromone may be present on the surface of the eggs. Experiments to test this hypothesis where carried out and organic solvent was used to remove any chemical present on the surface of the eggs. These experiments confirmed that a chemical was present on the surface of the eggs and that it could be transferred to an alternative media such as filter paper and others. Again these results are similar to studies on *Lu. longipalpis* and *P. papatasi*, carried out by others (El Naiem and Ward, 1991; Srinivasan *et al*., 1995) and establish the presence of an oviposition pheromone on the surface of eggs of this species also.

In the study reported here the effect of other semiochemicals from the oviposition site (kairomones) and their possible interaction with the oviposition pheromone were not done. It would be interesting in the future to see how these semiochemicals, which might be derived from decaying organic material e.g. faecal or other organic material influence oviposition behaviour. Very little is known about oviposition in sand flies in the wild, and as far as I am aware there have only been a small number of publications on oviposition in *Lu. longipalpis* and none that describe oviposition in *P. argentipes* in the wild or in the laboratory. The small amount of literature that is available suggests that sand flies oviposit in sites rich in decaying organic material therefore one might reasonably expect that odours from these sites might influence *P. argentipes* sand flies decision to oviposit and that these compounds might be attractants, stimulants or even deterrents. For example, when gravid *Lu. longipalpis* females were exposed to the oviposition

pheromone of that species together with extract of rabbit faeces, females were found to lay their eggs earlier and in greater numbers (El Naiem and Ward, 1992a; Dougherty *et al*., 1995). These authors established that the rabbit faeces extract was an oviposition stimulant and attractant. Dougherty *et al*. (1995) postulated that gravid female sand flies were directed to the oviposition sites, first by long range volatile attractants and by the physical quality of the oviposition substrate. They postulated that the oviposition pheromone is the final phase in oviposition site selection because it is believed that the pheromone influences gravid females over a very limited range. In *P. papatasi*, Schlein *et al*. (1990) demonstrated that the optimal condition chosen by gravid female would be based on physical and chemical cues of the oviposition substrate.

The study of oviposition in *P. argentipes* has demonstrated the presence of an oviposition pheromone on conspecific eggs. However, the chemical involved has not been unidentified. Further studies will be needed to identify the chemical structure of the *P. argentipes* oviposition pheromone and also of the possible oviposition attractants or stimulants. However it is very likely that in *P. argentipes* the pheromone is a medium chain length fatty acid as in *Lu. longipalpis* where it has been shown to be dodecanoic acid (Dougherty and Hamilton, 1997). This is important for future study in which with the knowledge, exploitation of using the synthetic oviposition pheromone together with the oviposition attractants or additives in the endemic area of visceral leishmaniasis (VL) will indirectly reduce the population of adult *P. argentipes* by attracting and killing the gravid females while ovipositing their eggs in their breeding areas.

6.1.2 Male Sex Pheromone Mediated Mating Behaviour

The behavioural bioassays described in Chapter 4 were carried out to determine if male *P. argentipes* produce a sex pheromone. Simple preliminary experiments showed that pairs of 6d virgin male and female *P. argentipes* mated more frequently than pairs of younger age categories (1, 2, 3, 4 and 5d). This could have been because the females were most receptive at that age or the males were most attractive at that age. From these preliminary studies it was decided to determine the response of 2d and 6d females to males of the same age (and 2d females to 6d males and vice-versa) in a simple moving air two-choice olfactometer (Y-tube). From these experiments it was clear that 6d males produced a sex pheromone (2d males did not) and 6d virgin females were more responsive to the sex pheromone than 2d females. Jarvis and Rutledge (1992), working with *Lu. longipalpis*, showed that mating success was correlated with male age; middle-aged males (6-10d) were more successful in obtaining mating than young (1-5d) or old (11-15d) flies. Jones (1997) also showed that mating success in *Lu. longipalpis* was age-dependent. She showed that females prefer to mate with middle-aged males rather than younger or older males. *P. argentipes* females may use the same mate choice strategy as *Lu. longipalpis* females are attracted to the vicinity of a group of males by the male produced sex pheromone and mate choice by the female is the result of a combination of male signals including the pheromone. In the Y-tube olfactometer experiments described in Chapter 4 the display behaviour of males was eliminated showing that the sex pheromone is important in attracting females, however these experiments could not determine

the importance of sex pheromone production by individual males. This would be an interesting and important experiment to carry out but however because of limitations in apparatus it is currently impossible to relate female choice to quantity of pheromone produced by individual males. The closest approach to answering this question was work carried out by Jones and Hamilton (1998), who showed that the successful male, in a competing pair of males, had more pheromone in his glands and spent more time wing-flapping than unsuccessful males. The limitation of that study is that the amount of pheromone in the glands may not be related to the amount of pheromone produced by the successful male.

Bioassays carried out in the presence of host odour showed that the presence of host odour was important in improving the response of virgin females to males. In *Lu. longipalpis* host kairomones added to the sex pheromone in a bioassay synergised the response of the females (Bray and Hamilton, 2007) and similar observations have been made of the effect of host odour on the attractiveness of synthetic sex pheromone in the field (Bray *et al.*, 2010). One possible scenario in *P. argentipes*, mating is that, males and females are attracted to a host animal first by host odour (kairomone) over long distance then the males disperse their sex pheromone (which is attractive over short range) while wing-flapping to attract females for mating. Of course there are many possible alternative explanations for the role of the sex pheromone and how it interacts with the host odour and until the experiments are done the precise role played by the sex pheromone will remain uncertain.

In my bioassays a bigger group of *P. argentipes* males was more attractive than a smaller group of males. Work by Palit *et al*. (1993) and Lane *et al*. (1990) showed that the ratio of males to females in wild leks ranged from 9:1 to 27:1; and 19:1, respectively, suggesting that bigger groups of males might be more attractive than smaller groups.

Females *P. argentipes* were more attracted to an organic solvent extract of males in the presence of host odour compared to when the extract was presented without host odour. As this response mimics the response seen by females to live males it adds weight to the conclusion that there is a chemical compound in the body of males that could attract females for mating. It was not possible to extend the investigation to analyse male extracts or male extracts plus host odour by G/MS or electrophysiology to determine the exact nature of the male sand fly produce chemical.

6.1.3 Courtship Behaviour of *P. argentipes*

Laboratory observations of pairs of *P. argentipes* males and females described in Chapter 5 established in detail the behaviours that are displayed by both sexes during courtship and their sequence. Some of the behaviours displayed by *P. argentipes* males (i.e. short hops, swaying of terminalia and wing flapping) have previously been observed during mating on or around the host during laboratory and field observations of *P. argentipes* and *Lu. longipalpis* sand flies (Ward *et al*., 1988; Lane *et al.*, 1990; Palit *et al*., 1993). Although stationary

wing flapping is commonly displayed by both *P. argentipes* males and females throughout mating it was not found to be an indicator of successful mating. Male wing flapping is thought to be associated with sex pheromone dispersal by males in other species of sand flies such as *Lu. longipalpis* (Lane *et al*., 1985; Ward *et al*., 1988; Jones and Hamilton, 1998). Although there is no direct evidence, Ashford (1974) suggested that wing flapping was related to sex pheromone production in *P. orientalis* and Lane *et al*. (1990) and Palit *et al.* (1993) suggested that it may serve a similar function in *P. argentipes.* Stationary wing flapping is apparently a common act in sand fly courtship but it is not an assurance of a successful mating.

Even though, “touching” and “facing” were two behaviours that were displayed quite frequently in both *P. argentipes* males and females, their functions are difficult to discern. In other insects e.g. *Drosophila melanogaster* touching is an act that allows exchange of information on the chemical compounds present on the insect cuticle. These chemicals, known as cuticular hydrocarbons, are involved in the mating process (Casares, 2007). Also in the long-horned beetle, cuticular hydrocarbons that are perceived by contact have been demonstrated to mediate mate recognition (Barbour *et al*., 2007). Studies of cuticular hydrocarbons in sand flies, especially in *Phlebotomus* species, including *P. argentipes*, have been done usually to differentiate between different populations and species (Kamhawi *et al*., 1992; Gebre-Michael *et al*., 1994; Mahamat and Hassanali, 1998). Though there is a lack of evidence showing that cuticular hydrocarbons are exchanged during this touching process it is possible that males touch females or females touch males

as a signal for mating or mate recognition or to distinguish potential mates. Whilst “facing” could simply be a physical act used to visually assess a potential mate. In a successful mating attempt, *P. argentipes* males approach females by flapping their wings, then when they are closely to females, they bend their abdomen laterally in an attempt to copulate, these behaviours have been referred to as a ‘courtship dance’ (Palit *et al*.,1993).

P. argentipes males displayed two behaviours (“circling and dipping” and “dipping”) that indicated that mating would not occur, significantly, these behaviours have not been reported in similar studies carried out with either in *P. papatasi* or *Lu. longipalpis*. Whenever a male displayed these behaviours, it indicated that the male was not interested in mating with his partner. Although an explanation for males rejecting females is difficult to perceive it may be that the male may have ejaculated his sperm while he dipped his terminalia on the surface of the arena or potentially the available partner was not compatible due to the male being unable to recognise his mate because of intrinsic factors such as age, body size, hormonal stage, mating status. Alternatively, the influence of external factors (such as surroundings, temperature, etc.) could affect male behaviour towards his mate (extrinsic behaviour). All of the observations for this study were carried out in a small observation chamber. In the work for Chapter 4, it was shown that the small observation chamber resulted in fewer successful copulations that a larger Barraud cage suggesting that the sand flies are constrained to an extent by their immediate surroundings. It would be ideal to repeat these male/female interactions in the larger arena to determine the

significance of these behaviours in a different environment. Darwin (1871) hypothesised that intrasexual competition (involving competition within one sex for individuals of the opposite sex) and mate choice (also known as intersexual selection involving preferential choice by one sex for individuals of the opposite sex) are the two mechanisms that stimulate sexual selection. Intrasexual competition normally refers to male-male competition that competes for the female to be his mate, whereas mate choice is more of a female's choice of attractive males. Few studies have examined potential costs of female choice and factors intrinsic to females that affect choice. Gray (1999) found that in female house crickets, *Acheta domesticus* age had a significant effect on female choosiness in which young females were selective of her mate but not older females. He also found that nutritional condition, body size, and size-relative reproductive investment did not influence female choice. Furthermore, females spent more time in choosing attractive males compared to unattractive males.

It is possible that *P. argentipes* employ not only chemical signals (e.g. the sex pheromone that is dispensed while "wing-flapping" and a contact pheromone while "touching") during courtship but also a physical (tactile) signal (also while "touching") and visual (when "facing"). In nocturnal Lepidoptera, females emit a species-specific sex pheromone to recruit a sexual partner (Shorey, 1973). This sex pheromone elicits an immediate response of sexual behaviour in mature, conspecific males, who exhibit a positive response in search of the pheromone source (Kennedy *et al*., 1981). When a male approaches the female, in response to a high pheromone concentration and potentially other cues, he is stimulated to

display his courtship behaviour, which may consist of chemical signals, with the extrusion of scent glands such as hair pencils (Birch *et al*., 1990), a series of contact moves (Girling and Cardé, 2006) and/or acoustic signaling by wing-fanning (Spangler, 1987). Female emission of and male response to pheromones are affected by extrinsic factors (e.g. temperature, wind speed, relative humidity) and intrinsic factors (e.g. age).

Mating behaviours are complex and involve a series of interdependent events including searching for mates, participation in courtship, copulation and the need to recover from the cumulative costs of previous events. Searching for mates and courtship involves communication and coordination between individuals including those that may not necessarily share similar intentions (Thornhill, 1979; Parker, 1983; Bradbury and Vehrencamp, 1998; Johansson and Jones, 2007). Therefore courtship behaviour is seen to be a complex hierarchical series of steps leading toward successful conspecific mating in many species of insects and the work presented here indicated that this is likely to be the case also for *P. argentipes*.

6.2 FUTURE WORK

The experiments that were carried out have demonstrated that pheromones are used to mediate oviposition and mating in *P. argentipes*. The pheromones are produced by *P. argentipes* females and males respectively, and their presence

demonstrated through oviposition and mating bioassays. The work presented here also investigated some aspects of the sequence of event involved in successful courtship behaviour. The work also showed that host odour cues (kairomones) are important and enhance the activity of the sex pheromone during mating. These findings have improved our knowledge of oviposition and mating behaviour in *P. argentipes* and raise the possibility of applying the knowledge for the control and monitoring of sand flies in a vector control programme.

Further work is required to improve and extend the current findings. In the case of oviposition pheromones of gravid *P. argentipes* females, although there was evidence showing that an oviposition pheromone helps to facilitate the choice of oviposition site, it is unclear if the effect of this oviposition cue is as a stimulant or attractant. Testing the *P. argentipes* oviposition pheromone in a moving air olfactometer such as a wind-tunnel or Y-tube might allow us to determine if it is an attractant or stimulant. In addition testing the oviposition pheromone in combination with environmental oviposition cues from frass, larval rearing medium or rabbit faeces, etc. might help reveal important information about the interactions between environment and insect. In nature, it is known that oviposition pheromones and other cues; such as environmental, physical and visual, will be used by gravid insects to find suitable oviposition sites to oviposit.

Identification of the chemical structure of the oviposition pheromone of *P. argentipes* and other oviposition attractants/stimulants would be a significant

addition to our understanding of *P. argentipes* oviposition behaviour and offer new opportunities for vector control. Further experiments to identify the chemicals involved and their interaction need to be done in the laboratory and field before we can make progress in the possibility of developing an oviposition trap to monitor or control populations of *P. argentipes*. However as ovitraps, with or without the addition of attractants in lures, have been used as one of the tools to reduce mosquito populations there is considerable potential for developing this line of work in sand flies.

The work in this thesis has established that *P. argentipes* male sex pheromone attracts *P. argentipes* females and the presence of host odour synergises the attraction. Further work is needed to identify and fully characterise the chemical compound(s) that make up the sex pheromone and the compounds in host odour that synergise it. In *Lu. longipalpis* species complex several novel methylsesquiterpenes were identified as the active component of the members of the complex. Each member has its own unique chemical. A preliminary examination of *P. argentipes* male extract did not reveal the presence of a similar class of compounds so further work to identify the chemical will involve complex statistical comparisons of male extracts and related electrophysiology to identify receptor active compounds. Further work to understand the basic biology of this communication system is also required. For example the range of concentrations and distances over which the chemical is attractive and more information on the age of males that produce the pheromone as well as the receptivity of females is should be explored in the laboratory and in the field.

Although the basic elements of the courtship behaviour of *P. argentipes* have been established, there is still more information that need to be gathered to fully understand the mating system; for example what is happening when the males and females have more space to move around in while making their choice. Further work to determine which of the individual courtship behaviours (if any) are exclusive and their exact function in regulating mating success may include the manipulation of the individual behaviours. Play-back experiments, for example, where the sounds that males and females make while mating, are included in a manipulative experimental setting, may reveal the importance of individual acoustic elements of the mating interactions.

A fuller understanding of the mating system of *P. argentipes* may help to develop the potential of this pheromone for its use for vector control. Several possibilities exist; 1) a 'lure-and-kill' strategy as is being developed currently against *Lu. longipalpis* in Brazil which comprises the use of a synthetic pheromone and animal host to attract females to a trap or insecticide (Bray *et al*., 2010) and; 2) mating disruption strategy in which release of synthetic sex pheromone over a wide area can prevent males and females from locating one another.

The extension of the preliminary studies described in this thesis will enhance and widen our knowledge on the biology, ecology and chemical mediated behaviour of *P. argentipes* that could lead to improve the efficiency and efficacy of

the sand fly control programmes, when used alongside existing insecticides, in which could directly reduce the amount of VL being transmitted among the populations at risk.

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APPENDIX A:

Peer-reviewed research article resulting from Chapter 5 of this thesis:

i) **Bray *et al*., 2014;**

Bray, D. P., Yaman, K., Underhill, B. A., Mitchell, F., Carter, V. and Hamilton, J. G. C. 2014. Multi-modal analysis of courtship behaviour in the old world leishmaniasis vector Phlebotomus argentipes. *PLOS Neglected Tropical Diseases, 8(12), e3316.*
DOI: 10.1371/journal.pntd.0003316

(Item follows on subsequent pages)

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PLOS | NEGLECTED TROPICAL DISEASES

Multi-modal Analysis of Courtship Behaviour in the Old World Leishmaniasis Vector *Phlebotomus argentipes*

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Abstract

Background: The sand fly *Phlebotomus argentipes* is arguably the most important vector of leishmaniasis worldwide. As there is no vaccine against the parasites that cause leishmaniasis, disease prevention focuses on control of the insect vector. Understanding reproductive behaviour will be essential to controlling populations of *P. argentipes*, and developing new strategies for reducing leishmaniasis transmission. Through statistical analysis of male-female interactions, this study provides a detailed description of *P. argentipes* courtship, and behaviours critical to mating success are highlighted. The potential for a role of cuticular hydrocarbons in *P. argentipes* courtship is also investigated, by comparing chemicals extracted from the surface of male and female flies.

Principal Findings: *P. argentipes* courtship shared many similarities with that of both *Phlebotomus papatasi* and the New World leishmaniasis vector *Lutzomyia longipalpis*. Male wing-flapping while approaching the female during courtship predicted mating success, and touching between males and females was a common and frequent occurrence. Both sexes were able to reject a potential partner. Significant differences were found in the profile of chemicals extracted from the surface of males and females. Results of GC analysis indicate that female extracts contained a number of peaks with relatively short retention times not present in males. Extracts from males had higher peaks for chemicals with relatively long retention times.

Conclusions: The importance of male approach flapping suggests that production of audio signals through wing beating, or dispersal of sex pheromones, are important to mating in this species. Frequent touching as a means of communication, and the differences in the chemical profiles extracted from males and females, may also indicate a role for cuticular hydrocarbons in *P. argentipes* courtship. Comparing characteristics of successful and unsuccessful mates could aid in identifying the modality of signals involved in *P. argentipes* courtship, and their potential for use in developing new strategies for vector control.

Citation: Bray DP, Yaman K, Underhilll BA, Mitchell F, Carter V, et al. (2014) Multi-modal Analysis of Courtship Behaviour in the Old World Leishmaniasis Vector *Phlebotomus argentipes*. PLoS Negl Trop Dis 8(12): e3316. doi:10.1371/journal.pntd.0003316

Editor: Paulo Filemon Pimenta, Fundaçao Oswaldo Cruz, Brazil

Received July 25, 2014; **Accepted** October 3, 2014; **Published** December 4, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: DPB, JGCH, VC and BAU were supported by the Wellcome Trust (grant 091689); FM and the GC/MS facility were supported by the Wellcome Trust (grant 089342); http://www.wellcome.ac.uk. KY was supported by a Malaysian Government PhD studentship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Visceral leishmaniasis (VL) is a debilitating disease estimated to cause 20,000–40,000 deaths worldwide each year [1]. The Indian subcontinent is one of the areas most affected by VL, with over 140,000 cases per year estimated to occur in India alone [1]. The etiologic agent in this region is the protozoan parasite *Leishmania donovani* (Kinetoplastida: Trypanosomatidae), with the sand fly *Phlebotomus argentipes* (Diptera: Psychodidae) the proven or suspected vector in Bangladesh, India, Nepal and Sri Lanka [2]. As there is no vaccine against VL, and cost and drug resistance limit effectiveness of treatment in India [3], control of the sand fly vector remains a priority for reducing transmission [4]. To be successful these programmes require a thorough understanding of the behaviour of the insect vector [5], not least because many human activities can significantly alter sand fly behaviour and potential risk of transmission. Agricultural practices, for example, may lead to creation of new habitats for sand flies [6]. Insecticide spraying for control can lead to unintentional diversion of sand flies away from normal resting sites in animal houses, potentially increasing the biting risk to humans [7,8].

Studies of insect vector mating behaviour facilitate development of novel tools for control. For example, a new approach for controlling the South American vector of VL, *Lutzomyia longipalpis*, exploits attraction to male-produced sex pheromones. A synthetic version of this chemical attracts both females and males

Author Summary

The sand fly *Phlebotomus argentipes* transmits *Leishmania* parasites through female blood-feeding. These parasites cause leishmaniasis, a potentially fatal disease for which there is no vaccine. Understanding how insect vectors behave can aid in developing strategies to reduce disease transmission. Here, we investigate courtship behaviour in *P. argentipes*. Courtship is critical to an organism's life cycle, as it is essential for mating and reproduction. We show that courtship in this species begins with the male wing-flapping while approaching the female. This behaviour may suggest production of audio signals, or dispersal of chemicals from the male, which the female finds attractive. There then follows a period of touching between males and females prior to copulation. This behaviour may function in the transmission and reception of chemical signals, present on the insect surface. Many insects use these kinds of chemicals in courtship, and here we show differences in the chemicals extracted from the cuticle of male and female *P. argentipes*. Both males and females were found to be able to reject a potential mate. Understanding why some *P. argentipes* are more attractive than others could help identify the signals essential to reproduction, and their potential for use in vector control.

to traps and insecticide-sprayed surfaces for up to 3 months in the field [8,9]. Field and laboratory observations suggest that *P. argentipes* shares some underlying behavioural characteristics with *L. longipalpis*. In both species, males form aggregations on or above host animals prior to the arrival of females, where mating and blood-feeding takes place [10,11]. Currently, very little is known about the signals which mediate male-female interactions in *P. argentipes*. Insect courtship is often a complex process, and can include transmission and reception of auditory, physical, visual and chemical signals between potential mates [12]. In common with *L. longipalpis*, aggregating male *P. argentipes* perform wing-flapping behaviours, but their relevance to mating or courtship is unknown [10,13–16]. There is also evidence that female *P. argentipes* investigate unidentified chemicals that can be extracted from male *P. argentipes* [13]. Hydrocarbons present in the cuticular wax, which function as chemical signals in mating behaviour of many insect species [17], have also been reported from many species of sand fly, including female *P. argentipes* [18]. However, the extent to which male and female *P. argentipes* differ in the hydrocarbons they produce, and how these potential chemicals signals might be transmitted during courtship (e.g. through touching [12]), remains to be investigated.

To date, studies of mating behaviour in *P. argentipes* have been limited to observations of aggregations on host animals [10,13,14]. The small-scale interactions between individual males and females, which occur prior to copulation, have not been described. The aim of this study was therefore to provide a detailed analysis of the individual behaviours performed by male and female *P. argentipes* during courtship, and the sequence in which they occur. Behaviours which predicted copulation success, and are therefore critical to mating, were identified through statistical analysis. Courtship in *P. argentipes* was then compared with that of *L. longipalpis* [16] and *Phlebotomus papatasi* [19], species from which there is also evidence of chemical communication [20]. Through a combination of gas chromatography and mathematical analysis, we also determined whether there are sex-specific differences in the chemicals present in or on the male and female cuticle of unmated *P. argentipes*. Such chemicals might play a crucial role in sexual signalling of this important disease vector.

Methods

Sand fly rearing

P. argentipes were from a colony maintained at Keele University, UK, for approximately 28 generations. Adults were kept in Barraud cages at 27°C, 95% RH, under a 12:12 light:dark photocycle. Females were blood fed 3 days post-emergence in accordance with UK Home Office Licence requirements (see Ethical Statement). Male and female *P. argentipes* used in both mating trials and chemical analyses were placed into single-sex cages within 5 h of eclosion (prior to rotation of male genitalia) to prevent mating prior to experiments, and fed only on saturated sugar solution.

Recording of courtship behaviour

Courtship interactions between 38 pairs of male and female *P. argentipes* were recorded under white florescent light in a purpose built bioassay room at Keele University. The males and females used were between 4 and 6 days old as this is the age at which they are believed to be sexually mature. The room was maintained at 27°C±2°C and 85% rh, with all recordings made between 1400 and 1800 hours. Courtship took place in a round plastic mating arena (22 mm ID×15 mm H) (Figure S1 and S2). The top of the arena was covered with a glass slide (76×26×1 mm) which prevented flies escaping while enabling videoing of courtship behaviour. Recordings were made using a colour video camera (TK-1280E; JVC, London, UK) fitted with a zoom lens (Computar 18–108 mm, f 2.5 manual focus; CBC (Europe) Ltd, London, UK) and supported 30 cm above the courtship arena using a copy stand (CS-920; Tracksys Ltd, Nottingham, UK). Output from the camera was fed through a vertical interval time code (VITC) generator (AEC-BOX-18; Adrienne Electronic Corp., Las Vegas, NV, USA) to a time-lapse security video recorder (VCR) (HS1024; Mitsubishi Electric, Hatfield, UK) set to non-stop recording. A feed from the VCR was sent to a colour monitor (Trinitron KV-14MIU; Sony, Thatcham, UK) to enable camera adjustments and observations while filming. Additional illumination for recording was provided by a fibre optic light source (KL 500; Schott UK Ltd, Stafford, UK).

For each observation, a male fly was placed into the arena, via a round hole made in the side, using a mouth aspirator. After a period of 5 min, the VCR was set to record and a female was placed into the arena using the aspirator. Males were placed into the arena first to mimic the natural behaviour of *P. argentipes*, in which males aggregate on host animals prior to the arrival of females [10]. Each observation was recorded for a maximum of ten minutes, or terminated earlier once the pair had disengaged from copulation. The copulation arena was cleaned with hexane to remove any contaminating volatiles (VWR International Ltd, Leighton Buzzard, UK) and left in a fume hood for the hexane to evaporate prior to reuse. The glass slide was washed with 5% Teepol detergent (VWR International, Lutterworth, United Kingdom), distilled water and acetone (Sigma Aldrich, Gillingham, UK) between trials.

Analysis of courtship behaviour

Recordings of courtship behaviours were analysed using a PC fitted with a PC-VITC card (Adrienne Electronic Corp., Henderson, USA), running the Observer Base Package for DOS (Version 3.0) and Support Package for Video Tape Analysis (Version 3.1; Noldus Information technology, Wageningen, the

Table 1. Behaviours performed during *P. argentipes* courtship.

	Name of behaviour	Description
	Male & female behaviours	
1	Not courting	Sand fly remains stationary or moves around the arena without wing-flapping, facing or touching its courtship partner.
2	Stationary wing-flapping	Sand fly remains stationary and flaps both wings simultaneously. Flapping followed a pattern of small vibrations through a slight rotation of the wings followed by a large flap, in which both wings extended to an angle of 45–70° from the body.
3	Touching	Sand fly makes contact with its partner by touching with the tips of the legs or antennae. Contact was most often made with the partner's legs or antennae, and occasionally the abdomen.
4	Facing	Male and female remaining motionless while facing one another.
5	Dipping	Sand fly moves vertically by dipping its abdomen to touch the floor of the arena, often in a repeating pattern.
6	Circling and dipping	Sand fly positions its head towards the arena floor and dips the end of its abdomen while moving in a circle or semi-circle around the same spot. Movement occurred in both clockwise and anticlockwise directions.
7	Copulation	Male and female copulate with the tips of the abdomen joined and facing in opposite directions. Males often flapped their wings until female appeared to accept copulation. Females normally remained motionless but occasionally struggled during copulation.
	Male-only behaviours	
8	Abdomen bending	Male bends his abdomen laterally; swinging his terminalia to the left and right, often while female is nearby.
9	Approach-flapping	Male rigorously flaps his wings and steps towards female in an alternating repeating pattern.
10	Copulation attempt	From a position parallel to the female, male bends his abdomen in an attempt to make contact with the female genitalia, often while wing-flapping.

doi:10.1371/journal.pntd.0003316.t001

Netherlands). Videos of courtship were replayed on the VCR, with the output sent simultaneously to the PC-VITC card and the Sony TV monitor. Behaviour of both male and female *P. argentipes* was coded into mutually exclusive categories (in which only one of the behaviours listed in Table 1 could be performed by each fly at any given time) and entered into the Observer software via a sequence of key presses during video playback. Video images were replayed in slow motion, with key presses in Observer synchronised to the time code recorded onto the video by the VITC generator, as read by the PC-VITC card.

Raw data on the order and duration of behaviours performed during courtship were exported from Observer into R version 3.1 [21]. These data formed the basis of subsequent analysis of behavioural transitions (see below). Frequency or duration of behaviours performed by males and females were compared statistically using Wilcoxon signed rank tests. Fisher's exact test was used to establish which male and female behaviours occurred more frequently in successful and unsuccessful courtships, in order to identify behaviours which predicted mating success.

Analysis of behavioural transitions

A log-linear modelling approach in R was used to devise a statistical model of courtship behaviour in *P. argentipes* [16,19,22,23]. Chi-square tests first established whether there was a significant overall association between preceding and following behaviour in male-male, male-female, female-female and female-male behavioural transitions during courtship (Tables S1, S2, S3, S4), ignoring periods of not courting (Table 1, behaviour 1). To improve robustness of $\mathbf{X}^2$ tests, behaviours which occurred less than five times in rows or columns of transition tables were excluded from analysis. Adjusted residuals >1.96 in a no-effect model identified individual behavioural transitions which occurred significantly more likely than expected by chance in each table [24,25]. Significant transitions were joined together to form a kinetogram outlining the overall sequence of behaviours in *P. argentipes* courtship (Figure 1).

Chemical analysis of cuticular profiles

Volatile and non-volatile chemicals present on the surface of the cuticle or in glandular tissue of sexually mature (4–6 day old), unmated male and female *P. argentipes* were extracted by placing individual flies in glass vials containing 20 µl of hexane for 15 minutes [26]. Following removal of flies, vials were sealed and stored at −20°C until use. For gas chromatography–mass spectrometry (GC/MS) analysis, individual extracts were reduced to dryness at room temperature under nitrogen and then re-suspended in 2 µl hexane prior to injection. Samples were analysed via splitless injection (inlet temperature: 280°C) into an Agilent 7890A-5975C GC/MS (Agilent Technologies UK Ltd, Cheshire, UK) on a non-polar HP-5MS column. Oven temperature was maintained at 75°C for 5 min, before rising at 17°C min^{-1} and held at 310°C for 10 min. The carrier gas was hydrogen.

Gas chromatographs expressed as detector response over time from 24 male and 24 female sand flies were imported into R for analysis [27]. Chromatographs were aligned, and variation in baseline was removed using the ptw (parametric time warping) package [28]. Noise in chromatograms was reduced by averaging responses over 25 ms, and ignoring peaks below a threshold of 50000 in height. This resulted in a set of 39 peaks not present in control 'blank' samples for further analysis. Principle component analysis (Psych package [29]) was then used to extract and rotate components explaining underlying variation in the matrix of peak heights for the 48 flies. Linear discriminant analysis with jack-knifed predictions (Mass package [30]) was then used to determine the accuracy with which fly sex could be predicted from the scores assigned to each sand fly from the extracted components.

Ethics statement

Female *P. argentipes* were blood fed on anaesthetized laboratory mice. All work involving blood-feeding was carried out in the UK under UK Home Office licence 4003279 and was approved by the Home Office. The Keele University animal

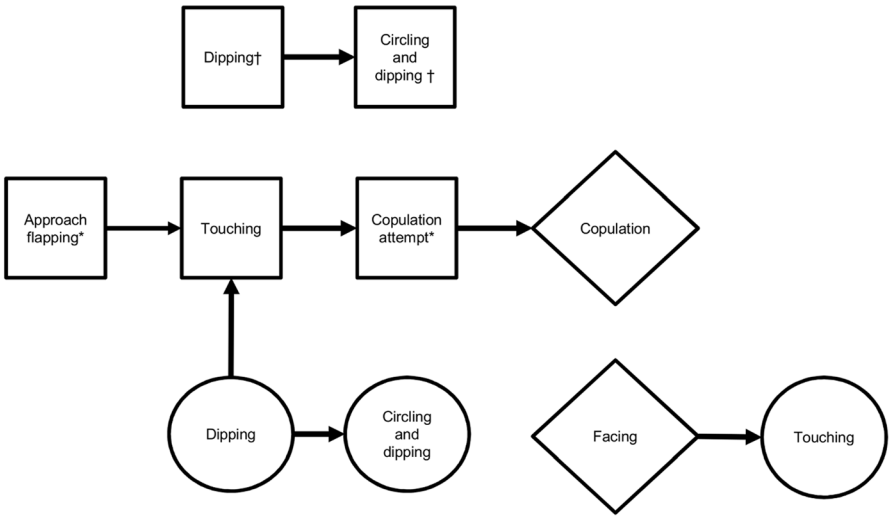

Figure 1. Kinetogram depicting sequence of male (square), female (circle) and joint (diamond) behaviours during *P. argentipes* courtship, based on observation of 38 male-female pairs. * Behaviour significantly (P<0.05) more likely to occur in successful courtships, ending in copulation. † Behaviour significantly more likely to occur in unsuccessful courtships (no copulation).
doi:10.1371/journal.pntd.0003316.g001

welfare ethics review board at Keele University also reviewed and approved the blood feeding protocol prior to commencement of this study. The study was conducted according to the guidelines set for animal husbandry by Keele University and the UK Home Office. These rules are governed by the Animals (Scientific Procedures) Act 1986. In addition we comply with the Common Rules for Animal Research that are prepared by the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

Results

Overview of courtship behaviour

Both male and female *P. argentipes* actively participated in courtship, performing stationary wing-flapping, touching, facing, dipping, and circling and dipping behaviours (Table 1). Males performed three behaviours not performed by females: abdomen bending (Table 1, behaviour 8), approaching the female while wing-flapping (behaviour 9) and attempting copulation (behaviour 10). Bouts of active courtship were separated by periods of not courting (behaviour 1), in which sand flies were either stationary or moving around the arena. In total, males spent a greater proportion of time during trials actively courting than females (median (25%–75% quartiles), males 20.5% (8.7%–35.1%), females 6.6% (3.0–20.5%), Wilcoxon signed rank test $P<0.01$).

While both sexes performed stationary wing-flapping (behaviour 2), males spent more time wing-flapping per trial than females (males: 27.8 s (8.2–90.6), females: 2.4 s (0.0–29.3), $P<0.001$), and wing-flapped more frequently (median behaviours per trial, males: 9.50 (3.0–17.6), females: 2.0 (0.0–10.0), $P<0.01$).

However, there was no difference between sexes in time spent touching per trial (behaviour 3) (males: 2.3 (0.2–6.5), females: 4.0 (0.0–7.6), not significant (NS)), or frequency of touching behaviours initiated per trial (males: 3.0 (1.0–5.8), females: 2.0 (0.0–3.0), NS). Similarly, there was no difference between sexes in time spent dipping (behaviour 5) (males: 0.0 (0.0–4.0), females (0.26 (0.0–8.1), NS) or overall frequency of dipping behaviours (males: 0.0 (0.0–1.0), females: 0.5 (0.0–2.0), NS). There was also no difference in time spent circling and dipping (behaviour 6) (males: 0.0 (0.0–0.0) [mean 3.1 s], females: 0.0 (0.0–0.0) [mean 4.7 s], NS), or frequency of circling and dipping (males 0.0 (0.0–0.0) [mean 0.3 behaviours per trial], females 0.0 (0.0–0.0) [mean 0.3], NS).

Pairs of sand flies spent a median of 2.5 s (0.6–3.7) facing (behaviour 4) in 15 of 38 trials in which this behaviour occurred. Males spent 2.3 s (0.76–4.0) approach flapping (behaviour 8), 1.8 s (1.2–5.2) abdomen bending (behaviour 9) and 0.7 s (0.3–1.7) attempting copulation (behaviour 10), where each of these behaviours occurred during courtship trials.

Courtship proceeded to copulation in 16/38 (42%) of the 10 minute trials. Where copulation occurred, median copulation latency (measured from the beginning of the trial) was 104.1 s (63.6–142.3). In ten cases, copulation was concluded within the 10 min trial, with a median duration of 264.4 s (81.4–315.4). Successful males copulated on their first (8 males) second (6 males) third (one male) or fifth (one male) attempt. Males were observed to continue wing-flapping during 4/16 (25%) copulations. In general, males flapped their wings rapidly when beginning copulation, but then ceased.

Sequence of behaviours during courtship

An overall effect of preceding behaviour on following behaviour was found in male-male behavioural transitions ($\mathbf{X}^2 = 168.7$, df = 48, $P<0.001$; Table S1). Significant individual transitions occurred between approach flapping and touching, touching and copulation attempt, and copulation attempt to copulation. A significant transition also occurred between dipping to circling and dipping. Similarly, an effect of preceding behaviour on following behaviour was also found for female-female transitions ($\mathbf{X}^2 = 45.5$, df = 11, $P<0.001$; Table S2). As for males, a significant transition occurred between dipping and circling and dipping. In addition, there was also a significant transition between facing and touching.

Examining behavioural interactions between sexes, an overall effect of preceding behaviour on following behaviour was found in male to female transitions ($\mathbf{X}^2 = 79.9$, df = 20, $P<0.001$; Table S3). Male copulation attempt led to copulation, and facing to female touching. An overall effect of preceding behaviour on following behaviour was also found in female to male transitions ($\mathbf{X}^2 = 34.3$, df = 20, $P<0.05$; Table S4), with the only significant individual transition occurring between female dipping and male touching.

Table 2. Behaviours predicting copulation during *P. argentipes* courtships.

	Unsuccessful courtships (n = 22)†	Successful courtships (n = 16)‡
Male behaviours		
Approach flapping	36.4%	81.3%**
Copulation attempt	9.1%	93.8%***
Abdomen bending	18.2%	50.0%
Circling and dipping	27.3%*	0.0%
Dipping	45.5%*	12.5%
Stationary wing-flapping	95.5%	100.0%
Touching	81.8%	87.5%
Female behaviours		
Circling and dipping	31.8%	6.3%
Dipping	59.1%	37.5%
Stationary wing-flapping	68.2%	62.5%
Touching	86.4%	56.3%
Joint behaviours		
Facing	45.5%	31.3%

†Percentage of unsuccessful courtships (no copulation) in which the behaviour occurs.
‡Percentage of successful courtships (copulation) in which the behaviour occurs. Asterisks indicate behaviours which occurred significantly more frequently in unsuccessful or successful courtships (Fishers exact test on count data: * P<0.05, ** P<0.01, *** P<0.001).
doi:10.1371/journal.pntd.0003316.t002

Behaviours predicting copulation

Two male behaviours, approach flapping (8) and attempting copulation (10) occurred significantly more frequently in courtships leading to copulation, and therefore predicted courtship success (Fisher's exact test, $P<0.05$, Table 2). Two further male behaviours, dipping (5) and circling and dipping (6) occurred more frequently in unsuccessful courtships than successful courtships. These behaviours may therefore signal rejection of the female as a potential mate. Occurrence of individual female behaviours or facing during courtship did not predict copulation (Fisher's exact test, $P<0.05$ Table 2).

Kinetogram of courtship behaviour

Analysis of behavioural transitions and occurrence of behaviours in successful and unsuccessful copulations suggests the following model of courtship in *P. argentipes* (Figure 1). In successful copulations, the male progresses from approach flapping, to touching, to attempting copulation, and copulation (Video S1). Dipping and circling and dipping appear to be related behaviours, and may indicate an unwillingness to mate. Female dipping was found to lead to the male touching the female, while periods of facing were followed by female touching the male. Both may indicate an attempt to investigate or prompt an unwilling mate.

Analysis of cuticular extracts

Two varimax-rotated principle components were extracted from the matrix of 39 peak heights derived from male and female *P. argentipes*. These two components explained 57% and 19% of the variation in peak height respectively. Plotting of component loading indicated that component 1 scaled positively with peaks with relatively short retention times (6.68–11.86 minutes; Figure 2, x axis). Component 2, scaled with peaks with relatively long retention times (13.54–21.79 minutes; Figure 2, y axis).

Plotting component scores for individual *P. argentipes*, females had higher scores for component 1, while males showed greater variation on component 2 (Figure 3). This translates to female extracts exhibiting higher peaks for chemicals with shorter retention times (which may not be present in males), and males higher peaks for chemicals with longer retention times (Figure 4).

Linear discriminate function analysis performed on the two rotated components resulted in jack-knife predictions of fly sex (male or female) which were significantly better than chance (fly sex correctly predicted in 75% of cases, Fishers exact test, $P<0.001$). Predictions for males (21/24, 88% of individuals correctly sexed) were more accurate than those for females (15/24, 63%). This difference in predictive ability may reflect the general absence of variation in males in component 1: i.e. peaks with low retention times present in females, but not males (Figure 4).

Discussion

Courtship behaviour in *P. argentipes* shared several similarities with both *P. papatasi* and the new world leishmaniasis vector *L. longipalpis*. The core progression of behaviours comprised the male wing-flapping while approaching the female, before touching her with the legs or antennae prior to attempting copulation. This builds on a previous description of the 'courtship dance' of *P. argentipes*, described as involving males hopping, swinging the terminalia and wing-flapping [14]. Both female and male *P. argentipes* engaged in wing-flapping behaviour during courtship, with male approach flapping a significant predictor of copulation success. While integral to *P. argentipes* courtship, the function of wing-flapping in this species is currently unknown. In *L. longipalpis*, male wing-flapping has been hypothesised to aid in dispersal of attractive sex pheromones released from abdominal tergites [15,31]. These pheromones attract female *L. longipalpis* to aggregations of males formed on or above host animals [32]. Male *P. argentipes* also form mating aggregations on cows or other animals, and perform wing-flapping behaviours prior to the arrival of females [10,13]. It is therefore possible that male *P. argentipes* also release an attractive sex pheromone to aid females in locating these aggregations. Male *P. argentipes* also performed abdomen

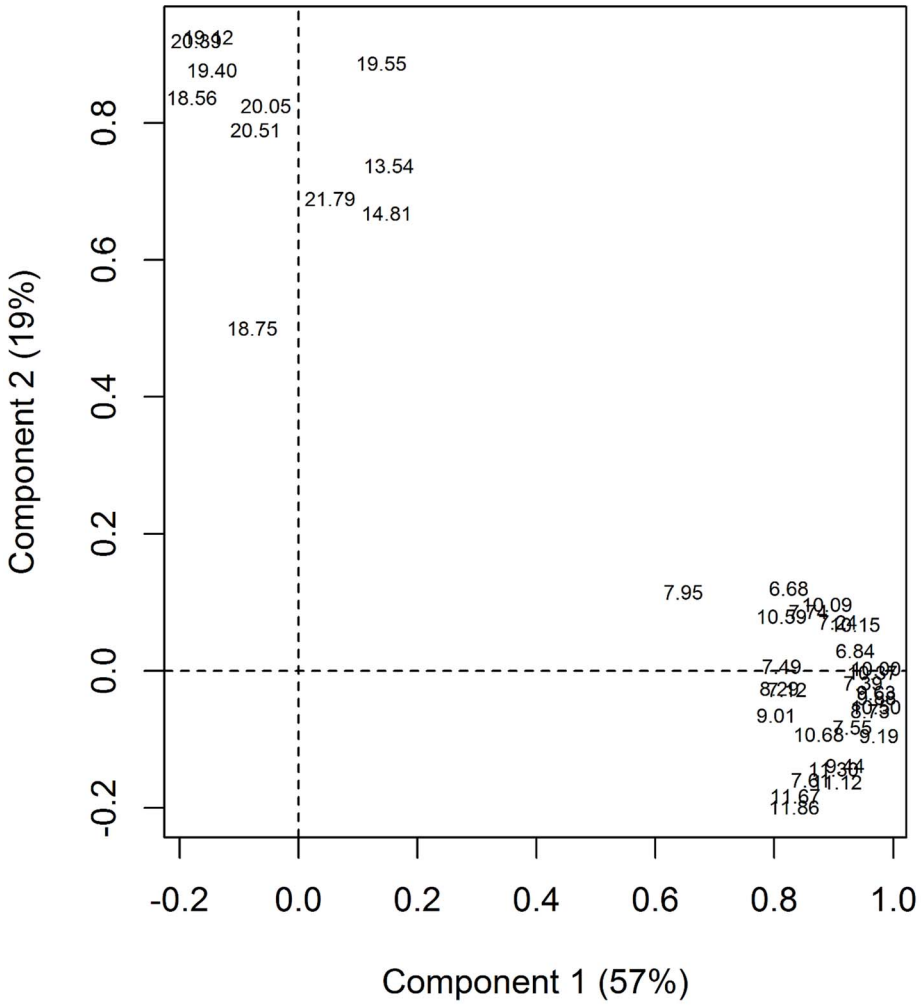

Figure 2. Component loadings for gas chromatogram peaks with different retention times extracted from 24 male and 24 female *P. argentipes*. Peaks with lower retention times had higher loadings for rotated component 1, which explained 57% of the variation in the original dataset. Peaks with higher retention times had higher loading for component 2, which explained 19% of the original variation.
doi:10.1371/journal.pntd.0003316.g002

bending during courtship, a behaviour previously reported from *Phlebotomus papatasi* [19], *Phlebotomus longipes* [33] *Phlebotomus martini* [34] and *Lutzomyia vexator* [35]. This could conceivably also function in pheromone release from abdominal tergites, the site of production of pheromones in *L. longipalpis*, [36]. There is behavioural evidence of chemically mediated attraction of females to males in both *P. argentipes* and *P. papatasi* [13,20]. However, to date no sex pheromone, or likely sex pheromone-producing structure, has been identified in any of the abdomen-bending sand flies [37], and *L. longipalpis* (which does produce pheromones) does not perform this behaviour [16].

In addition to chemical communication, *P. argentipes* wing-flapping may also function in production of audio signals important to mating. Courtship songs, produced by rhythmic wing vibrations are believed to play a role in species recognition in *L. longipalpis*, as the pattern of sound produced by males during copulation differs between members of the species complex [38,39]. Similar audio signals have also been recorded during courtship in *Lutzomyia intermedia* [40], and during copulation in *Lutzomyia cruzi* [41] and *Lutzomyia migonei* [42]. To our knowledge, no audio signals have been recorded from *P. argentipes*, despite descriptions of wing-flapping in this and other Old World species [19,43]. As in *P. papatasi*, male *P. argentipes* flapped their wings only briefly at the start of copulation [19], possibly to assist in alignment of the male and female genitalia. This may suggest that audio signals produced prior to copulation (rather than during) may play a greater role in courtship. Manipulative playback experiments, similar to those carried out in *Drosophila* [44] are needed to determine the function of audio signals (if any) in sand fly mating behaviour.

Whether associated with chemical, audio or visual signals, wing-flapping appears to be a predominantly male activity, with male *P. argentipes* wing-flapping more frequently, and for longer periods of time than females. The same trend has previously been observed in both *P. papatasi* and *L. longipalpis* [16,19]. In these species, female wing-flapping was found to be a predictor of courtship success, possibly indicating a willingness to mate. The same was not found to be the case of *P. argentipes* reported here.

Touching, initiated by both male and female *P. argentipes*, was frequently observed during courtship. This behaviour has also been reported from studies of *P. papatasi* and *L. longipalpis* [16,19]. Whilst found to be an integral part of the behavioural progression towards copulation, occurrence of this behaviour does not in itself predict copulation success in any of the three species

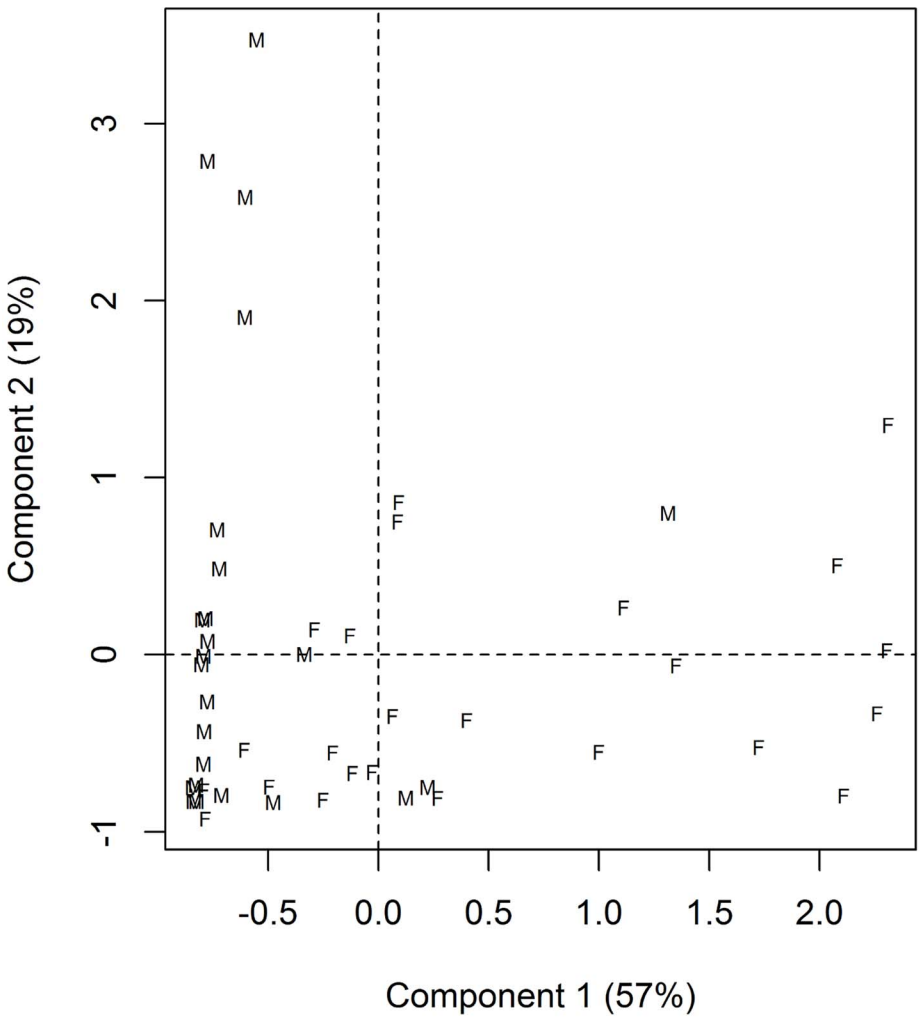

Figure 3. PCA component scores for 24 male and 24 female *P. argentipes*. Females had generally higher scores for component 1, while males showed greater variation in scores along component 2.
doi:10.1371/journal.pntd.0003316.g003

examined to date [16,19]. Touching in many insects, including member of the genus *Drosophila*, is involved in the transmission of short-range pheromones during courtship [12,45]. These chemicals include cuticular hydrocarbons, which can provide a range of information including species, sex, age and mating status of a potential partner [46]. Here, GC analysis revealed consistent differences in the profile of chemicals extracted from the surface of male and female *P. argentipes*. Comparison of retention times with straight chain alkanes suggest the recovered female-associated chemicals may be smaller than the C20–C40 chemicals normally recovered from cuticle wax [47]. The male-associated peaks however appear to be in the range for cuticular hydrocarbons, although identification will be required to confirm their structure. Previous studies have revealed variation in similar extracts from female *P. argentipes* from different regions, and between wild and colonized females [18]. Taken together, the results here indicate that there are also differences in the chemical profile of males and females, and that a potential behavioural mechanism exists for transmission and reception of these chemicals (touching). However, this is not in itself evidence for sex pheromones: more work is required to identify the potential chemicals involved, and to conduct bioassays to ascertain their relevance to mating and other behaviour. In particular, experiments are needed to determine whether the male-associated chemicals detected here could be responsible for the response of female *P. argentipes* to male extracts [13].

Courtship analysis revealed that male *P. argentipes* could signal an unwillingness to mate by dipping their abdomen toward the surface of the arena. When this occurred, copulation was significant less likely to occur. Similar abdomen dipping behaviour has previously been observed in female *L. longipalpis*, which are free to choose from a number of potential mates within a lek [15]. It has been suggested that in *L. longipalpis* this behaviour is linked to monandry as for the female the correct mate choice is essential. Why male *P. argentipes* should reject a potential mate is unclear, as males make relatively little contribution to offspring production. As only virgin males were used in this study, sperm depletion is also unlikely to explain this result. Further work is needed to ascertain whether rejection of females is a genuine feature of mating behaviour of *P. argentipes*, or an artefact of the trial conditions. If chemically mediated, mate rejection could form a target for mating disruption as a means of vector control.

Where mating did take place, *P. argentipes* copulated back to back, as occurs in most species of sand fly. There was no evidence

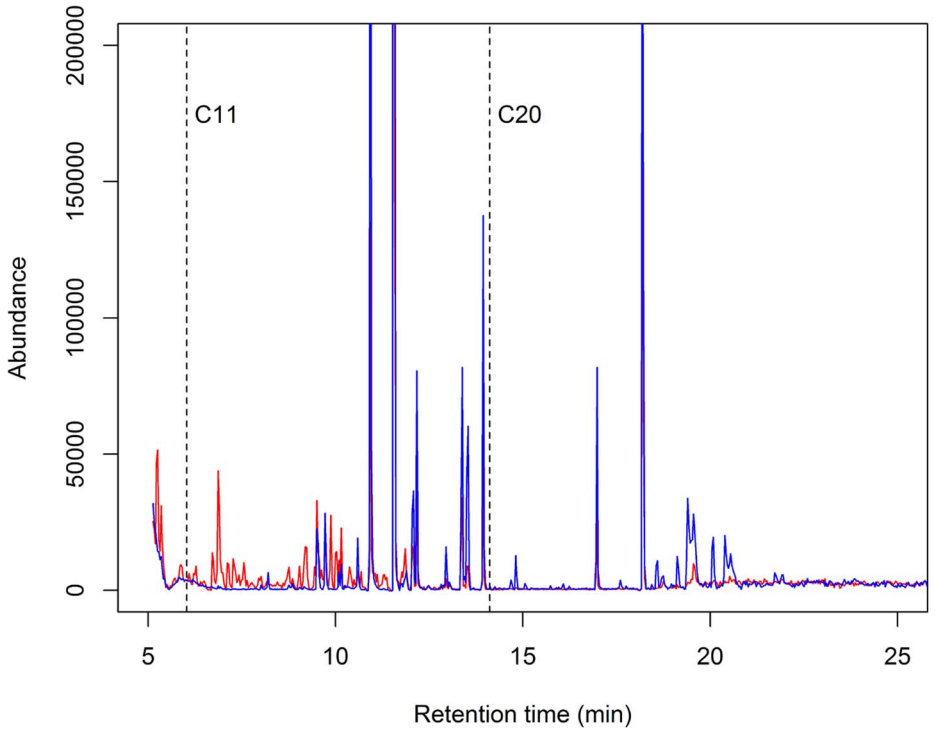

Figure 4. Example cleaned gas chromatographs extracted from individual male (blue line) and female (red line) *P. argentipes*. Females appeared to possess chemicals with lower retention times (less than 12 minutes) not recovered from males. Conversely males had larger peaks for chemicals present at retention times greater than 18 minutes. Dotted vertical lines represent retention times for undecane (C11) and eicosane (C20) under the same temperature programme.
doi:10.1371/journal.pntd.0003316.g004

of piggy backing behaviour (a possible mate-guarding activity), as performed by *Phlebotomus duboscqi* [48]. As in *L. longipalpis* and *P. papatasi*, there was considerable variation in both copulation latency, and the duration of copulation in *P. argentipes* [16,19]. The extent to which the latter is related to successful transfer of sperm and subsequent fertilization is unknown.

Very little is known about the mating strategy of *P. argentipes*. Experiments to answer questions such as whether females mate only once or more often, or why males appear to reject females are essential for developing control strategies. The results of this study demonstrate that courtship in *P. argentipes* shares similarities with both the new world VL vector *L. longipalpis*, and the Old World cutaneous leishmaniasis vector *P. papatasi*. As wing-flapping seems crucial to mating in this species, future studies should attempt to identify the modality of the signal produced by this behaviour, and its potential for exploitation as a means of vector control. Similarly, chemical analyses and behavioural bioassays are now required to identify the chemicals present on the surface of male and female *P. argentipes*, and to determine if they have any role in attracting or dissuading potential mates. Both sexes of *P. argentipes* reject potential mates, which suggests that some individuals are more attractive than others. *L. longipalpis* females are known to prefer a small number of males within an aggregation, and attractiveness in this species is both an inheritable characteristic, and associated with pheromone production [15,49]. Identifying differences between relatively attractive and unattractive individuals in *P. argentipes* would be a logical next step in identifying the modality of sexual signals used in this species, and their potential for exploitation in vector control.

Supporting Information

Figure S1 Close-up image of the arena used to observe male/female courtship interactions. The image shows the walls of the arena resting on a glass slide covered with a glass coverslip. For each observation, a male fly was placed into the arena, via a round hole made in the side, using a mouth aspirator.
(JPG)

Figure S2 Close-up image of the arena used to observe male/female courtship interactions. The image shows the walls of the arena resting on a glass slide covered with a glass coverslip. For each observation, a male fly was placed into the arena, via a round hole made in the side, using a mouth aspirator.
(JPG)

Table S1 Frequencies of male to male behaviours.
(DOCX)

Table S2 Frequencies of female to female behaviours.
(DOCX)

Table S3 Frequencies of male to female behaviours.
(DOCX)

Table S4 Frequencies of female to male behaviours.
(DOCX)

Video S1 Courtship behaviour of male and female *P. argentipes*. The thinner male, identifiable by the genital clasper at the end of the abdomen, approaches the female while wing-flapping (Table 1, behaviour 9), who also performs wing-flapping while

stationary (behaviour 2).The male makes contact with the female through touching with the legs or antennae (behaviour 3) several times prior to copulation.
(MP4)

Author Contributions

Conceived and designed the experiments: JGCH KY DPB. Performed the experiments: KY DPB BAU VC. Analyzed the data: DPB JGCH KY VC FM. Wrote the paper: DPB KY VC JGCH.

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